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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)
We have been studying a family of proteins that we have termed SIBLINGs for Small Integrin Binding Ligand N-linked Glycoproteins, that share similar structural domains, human chromosomal location, normal synthesis by skeletal tissue, and abnormal expression by neoplasms. The goal of the our research is to test whether SIBLINGs might be informative markers for breast cancer detection. To accomplish this goal we have developed competitive enzyme-linked immunosorbent assays (ELISAs) for the SIBLINGs bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein-1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE). Sandwich-based ELISA assays have also been developed. When the competitive ELISAs were used to screen SIBLING protein levels, BSP and OPN exhibited the highest degree of sensitivity and specificity for the detection of breast cancer. When SIBLING levels in breast cancer patient-derived samples were segregated grossly by tumor type (lobular versus ductal), statistically significant differences between the two groups were observed. Microarray analysis of normal and breast cancer-derived mRNA samples found a similar elevated levels of elevated SIBLING expression and differences by tumor type. These results suggest that SIBLINGs may have utility as serum-based markers for breast cancer detection.

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Introduction

Tumor progression involves modulation of cell adhesion, differentiation, division, apoptosis, angiogenesis as well as migration and metastasis. We have been studying a gene family we term SIBLINGs (for Small Integrin-Binding Ligand N-linked Glycoproteins) that are induced by certain neoplasms. Members of the SIBLING family include bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein (DMP1), dentin sialophosphoprotein, and matrix extracellular phosphoglycoprotein. Our published work has shown that BSP and OPN are extended and flexible in solution (such lack of ordered structure is shared by a number proteins that have multiple binding partners) [1]. SIBLINGs can bind integrins including $\alpha_v\beta_3$ via their RGD sequence [2-4]. OPN and DMP1 can also bind CD44 (via an amino terminal domain) [5-7]. SIBLINGs can bind to complement Factor H and sequester it to the cell surface thereby regulating complement-mediated cell lysis [7, 8]. More recently we have shown that SIBLINGs can bind to and modulate the activity of specific MMPs [9]. It is our hypothesis that SIBLINGs promote breast cancer progression through neoplastic expression of SIBLINGs that bind to and modulate the activity of specific MMPs. MMPs play multiple roles in tumor progression including: angiogenesis; processing and presentation of certain growth factors; and metastasis. We further hypothesize that SIBLINGs are biologically plausible surrogate endpoint markers for cancer detection.

The goal of the current research is to develop SIBLINGs as serum measures for use in breast cancer detection, by determining the distribution of their serum levels in a breast cancer patient population before and after treatment, a large normal (cancer-free) population, and a patient population at risk for developing breast cancer. Serum levels of gene family members in normal and breast cancer patients will be used to establish the sensitivity, specificity and predictive value of these markers in breast cancer. In patients with defined breast cancer, serum levels will be correlated with stage, prognosis and response to treatment. This research will determine whether serum SIBLING levels have high sensitivity (low false negative rate) and high specificity (low false positive rate), can be analyzed in a general laboratory setting (does not require highly specialized procedures/equipment), and enable early detection.

Body

The tasks outlined in the original Statement of Work for the first year were to:

Task 1. To complete development of competitive ELISA for the SIBLINGs DMP1 and MEPE (Months 1 – 6):

- a. Develop adenovirus expression vector for expressing recombinant human MEPE.
- b. Perform checkerboard assays to determine optimum antigen coating and antibody concentrations for MEPE and DMP1.
- c. Determine precision and yield of these new assays.

Task 2. To determine the distribution of serum SIBLING (BSP, OPN, DMP1 and MEPE) levels in serum obtained from normal donors and breast cancer patients (Months 7 - 24).

- a. Measure BSP, OPN, DMP1 and MEPE in a normal population, breast cancer patients and a population at risk for breast cancer.
- b. Determine sensitivity and specificity and perform ROC analysis.
- c. Test for clinical correlation between serum SIBLING levels and cancer stage, prognosis, tumor burden and response to treatment.

Task 3. To refine the existing competitive ELISA assay systems to more rapid sandwich-based assay systems and verify previous results (Months 24 – 36).

- a. Screen monoclonal antibodies for utility in OPN, DMP1 and MEPE assays.
- b. Employ checkerboard assays to define optimum capture antibody coating, second antibody concentration and incubation time.
- c. Re-analyze normal and breast cancer patient sera using the new sandwich based assays.

Progress.

Overview:

As of the end of the second year of this grant, Task 1 has been completed and both tasks 2 and 3 are well under way. As described in the previous annual report, the decision was made to carry out the competitive ELISA analysis while at the same time developing the sandwich-based assays. Work on characterizing the biology of SIBLING interactions with matrix metalloproteinases (MMPs) has also continued. A paper in the FASEB Journal describing this work has been published during the current reporting period and a second manuscript describing our characterization of MEPE levels in a large normal population is in press at the Journal of Clinical Endocrinology and Metabolism. Those two papers are included in the Appendix section as is a manuscript that is currently under review at Clinical Cancer Research that describes SIBLING expression in different cancers as well as associations between SIBLING levels and tumor stages.

During the current reporting period and on the strength of data generated from our studies of SIBLING biology, the P.I. successfully applied for Associate Membership in the Early Detection Research Network (EDRN) maintained by the National Cancer Institute. Membership in EDRN enables access to banked serum samples as well as quite extensive breast tumor tissue arrays. Also during this period, the P.I. has applied for funding through the National Institutes of Health to further pursue the studies of the interactions between SIBLINGs and MMPs and through the Department of Defense to develop the SIBLING DSPP as a biomarker for prostate cancer progression.

Detailed Progress Description.

A. SIBLING Biology. (Summary of Appendix I)

As described in the annual report for year one, during the course of purifying recombinant SIBLINGs for use in immunoassays, proteins that co-purified with specific SIBLINGs under nondenaturing conditions were observed. These proteins have subsequently been identified as matrix metalloproteinases (MMPs). MMPs are a class of hydrolytic enzymes defined by common structure and a requirement for zinc in the active site. Currently it is accepted that the inhibitory propeptide must be removed before the MMP can be enzymatically active. After the removal of the propeptide the MMP typically remains active until a TIMP (tissue inhibitor of matrix metalloproteinase) binds and inactivates the protease. In addition to their roles in tissue remodeling, MMPs play major roles in tumor cell progression and metastasis [10]. We have recently shown that at least three members of the SIBLING family specifically bind to different proMMPs and modulate their activity (Appendix I) [9].

This report demonstrated that three members of the SIBLING family (BSP, OPN and DMP1) were able to specifically bind ($K_d \approx nM$) three different MMPs. BSP specifically bound to pro- and active MMP-2, while OPN bound to pro- and active MMP-3, and DMP1 bound pro- and active MMP-9. Binding was demonstrated by: 1) co-purification of the pairs through nondenaturing chromatographic columns; 2) solution phase intrinsic fluorescence binding studies; and 3) showing that BSP and OPN affinity columns can be used to purify MMP-2 and MMP-3, respectively, from media containing several MMPs. Binding of SIBLING to their corresponding proMMPs was associated with structural changes as indicated by: 1) fluorescent quenching during SIBLING binding titration (indicating a change in the microenvironment of the MMP's tryptophans); 2) increased susceptibility of proMMP-SIBLING pairs to plasmin digestion; 3) increased enzymatic activity of the pro-MMPs upon binding their specific SIBLING partner; 4) reduced ability of specific low molecular weight inhibitors to block SIBLING + MMP complexes; and 5) restoration of activity to TIMP-inhibited MMPs by the corresponding SIBLING. These data suggested that the SIBLING family offers an alternative method of controlling the activity of at least three MMPs [9]

The biological activity that SIBLINGs possess (modulation of MMP activity through activation of the latent proenzyme and reactivation of TIMP inhibited MMP) is consistent with a role in early tumor progression [9]. Perhaps most significantly (from a clinical standpoint) SIBLINGs were found to restore activity to propeptide-free MMPs whose activity had been blocked by both natural and synthetic inhibitors. SIBLINGs are induced by neoplasms *in vivo* and their modulation of MMP activity (as demonstrated in this manuscript) might contribute to the relative lack of efficacy seen in the recent clinical trials of MMP inhibitors in numerous cancers.

B. Distribution and Levels of SIBLINGs.

As described in the year 1 annual report, all steps for Task 1 but the creation of a DMP1 immunoassay had been completed. During year 2 we have successfully developed a DMP1 competitive immunoassay (Figure 1). The competitive ELISAs for the five different SIBLINGs have been used to determine the levels and distribution in serum from normal donors (Figure 2).

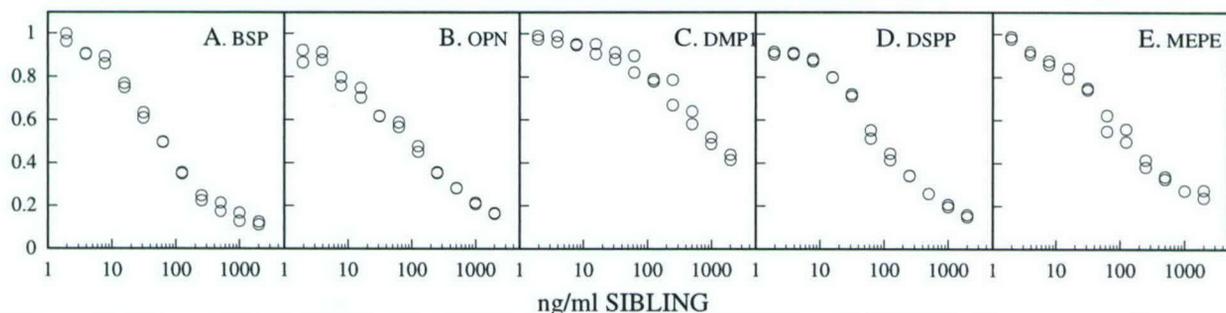


Figure 1. Competitive ELISAs for BSP, OPN, DMP1, DSPP and MEPE. Serum samples were diluted 1:10 in a 50% formamide 40 mM phosphate buffer, pH 7.4 and reduced with 2 mM dithiothreitol at 100 C for 5 min to disrupt the binding of BSP or OPN by complement factor H in serum. Residual reducing agent and formamide was removed by strong anion exchange spin column chromatography (ToyoPearl QAE resin, TosoHaas, Montgomeryville, PA) and samples were taken for analysis using a modified competitive ELISA. Greiner high binding plates were coated with 20 ng/ml BSP, or 20 ng/ml OPN, or 5 ng/ml DMP1, or 25 ng/ml DSPP, or 10 ng/ml MEPE overnight in 50 mM carbonate buffer, pH 8.0. Samples and standards were incubated for 2 h with shaking at room temperature with a 1:200,000 dilution of LF-100 antibody (for BSP) or 1:100,000 of LF-124 antibody (for OPN), or 1:200,000 LF148 (for DMP1), or 1:150,000 of LF-151 (for DSPP), or 1:200,000 of LF-155 (for MEPE) in a Tris buffered saline solution (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.05% Tween 20 (TBS-T) in polypropylene 96 well plates. LF antibodies were obtained from Dr. Larry Fisher, NIDCR, NIH [11]. During those 2 h the antigen coated plates were blocked with TBS + 5% powdered milk. Antigen coated plates were then rinsed three times with TBS-T and the antibody-sample solution added to the wells. After a second incubation for 1 h at room temperature with shaking the plates were washed three times with TBS-T. A secondary antibody of goat anti-rabbit peroxidase-labeled antibody conjugate, human serum adsorbed (Kierkagaard & Perry, Gaithersburg, MD) at 1:2000 was then added and the plates incubated for 1h. After three washes with TBS-T, substrate (3,3',5,5'-tetramethylbenzidine microwell peroxidase substrate, BioFX Laboratories) was added and after a final 20 min incubation the color reaction was stopped. Absorbance was read at 450 nm and the data analyzed using the program AssayZap (BioSoft, Cambridge, UK).

We have previously reported the normal distribution for BSP and OPN [12] and can now describe the results for DMP1 and DSPP. A paper in press and slated for publication in the August issue of the Journal of Clinical Endocrinology and Metabolism (included in Appendix) described the unique distribution pattern observed for MEPE in normal serum (see below). The SIBLINGs BSP, OPN, DMP1 and DSPP all exhibit a normal bell shaped Gaussian distribution to their values (Figure 2). In contrast, the distribution of MEPE was asymmetric (Figure 1d in

Appendix II) and when serum values were plotted as a function of donor age, an age-dependent decrease was observed (Figure 2a in Appendix II). None of the other SIBLINGs exhibited a significant age-dependent change in their serum values (data not shown).

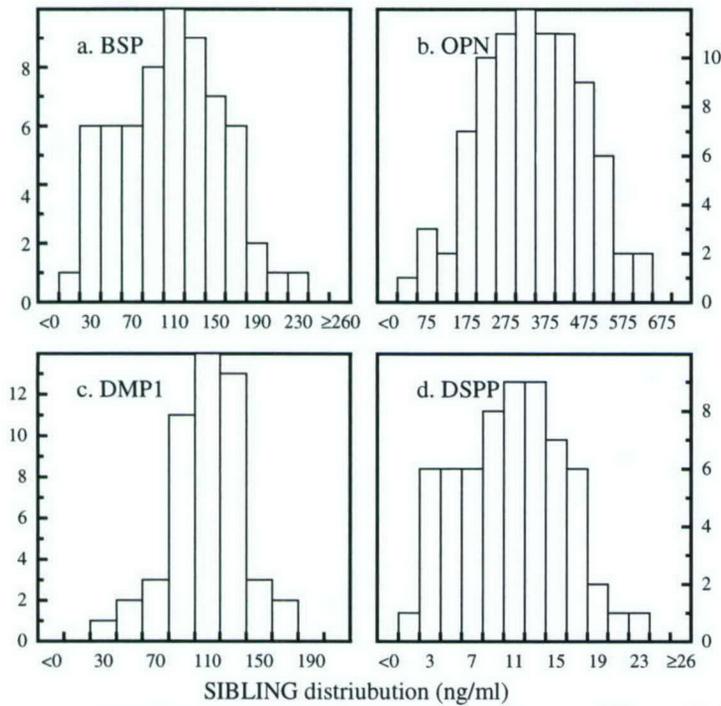


Figure 2. Distribution of serum BSP, OPN, DMP1 and DSPP levels in a normal population. Competitive ELISAs performed as described in Figure 1 were used to analyze serum levels of (a) BSP, (b) OPN, (c) DMP1, and (d) DSPP in normal donors. As part of the initial study design, breast cancer samples were paired with age-matched normal samples, though the results from the normal distribution analysis indicate that age-matching was not necessarily required (i.e. – BSP, OPN, DMP1 and DSPP exhibit no significant age-related changes in serum levels). To date, the number of paired normal and breast cancer samples analyzed are 63 for BSP and DSPP, 78 for OPN and 49 for DMP1.

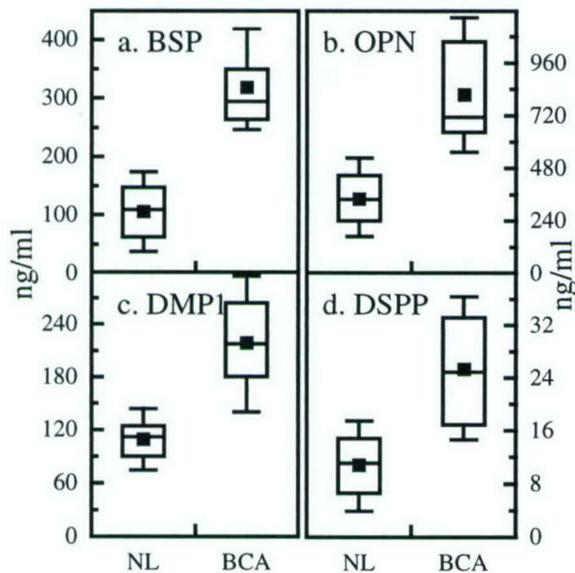


Figure 3. Levels of serum BSP, OPN, DMP1 and DSPP in a normal population and a population with breast cancer. Samples were analyzed by competitive ELISA (as described in Figure 1, above) and the mean values of duplicate analyses were plotted for (a) BSP, (b) OPN, (c) DMP1, and (d) DSPP by box plot. For the box plots, top, bottom and line through the middle correspond to 75th percentile, 25th percentile and 50th percentile (median), respectively. Error bar whiskers represent the 10th and 90th percentile, while the solid square symbol indicates the arithmetic mean. MEPE levels were no different between a normal population and a population with breast cancer (data not shown). Abbreviations, NL, normal; BCA, breast cancer.

C. Serum MEPE is uninformative for breast cancer, but does correlate with bone mineral density, parathyroid hormone and phosphorus. (Summary of Appendix II).

Normal MEPE expression has been described primarily in bone marrow, brain [13] and bone [14], while tumors which cause hypophosphatemic osteomalacia exhibit high expression and secretion [13]. Elevated levels of MEPE mRNA expression by tumors from patients with hypophosphatemia and osteomalacia suggested that it might be involved in mineral homeostasis. The control of systemic phosphate homeostasis is incompletely understood. Key modulators include PTH, calcium, phosphorus, vitamin D, as well as novel phosphatonin(s), and the bone and kidney organs. Candidate phosphaturic factors include MEPE; PHEX, a putative endopeptidase

believed to process factors regulating bone mineralization and renal phosphate reabsorption; FGF23, a phosphaturic factor in fibrous dysplasia, tumor-induced osteomalacia and autosomal-dominant hypophosphatemic rickets and secreted frizzled-related protein 4, an antagonist of renal Wnt-signaling [15].

In the paper by Jain et al. (Appendix II, [15]), we have shown that a) significant levels of MEPE in the serum of normal humans can be measured, (b) a clear age-related decrease in serum MEPE levels, (c) a positive correlation between MEPE and phosphorus, a inverse correlation with parathyroid hormone, and (d) a significant positive correlation with total hip and neck bone mineral density. While this study demonstrates the association of serum MEPE levels with serum phosphate, PTH and bone mineral density, it does not address causality. In the very least, the results suggest that MEPE may be an interesting marker of normal human bone and mineral metabolism.

D. SIBLING levels are elevated in multiple cancer types and correlate with specific MMP expression, tumor type and stage. (Summary of Appendix III).

This study was undertaken to determine the mRNA expression patterns of SIBLINGs in 9 different types of cancer. An additional goal was to determine whether SIBLINGs exhibited expression levels that correlated with their MMP partners as well as various measures of tumor progression. Cancer profiling arrays containing normalized cDNA from both tumor and corresponding normal tissues from 241 individual patients were employed to screen for SIBLING and MMP expression in 9 distinct cancer types.

Significantly elevated expression levels were observed for BSP in cancer of the breast, colon, stomach, rectum, thyroid and kidney; OPN in cancer of the breast, uterus, colon, ovary, lung, rectum, and thyroid; DMP1 in cancer of the breast, uterus, colon and lung; DSPP in breast and lung cancer. The degree of correlation between a SIBLING and its partner MMP was found to be significant within a given cancer type (e.g. BSP and MMP-2 in colon cancer, OPN and MMP-3 in ovarian cancer; DMP1 and MMP-9 in lung cancer).

SIBLING expression was different between different subtypes of cancer. While the historical basis for the distinction between the main two types of breast cancer (the belief that ductal carcinomas arose from ducts and lobular carcinomas from lobules) is subject to debate (both can arise from the terminal duct lobular unit), there is evidence that the two classes as used clinically refer to disease entities that differ in tumor size, shape, dissemination and proliferation rates [16]. The most common hallmark associated with the lobular classification is multifocality. Lobular tumors tend to be more slowly proliferating than ductal tumors. They also tend to frequently exhibit hormone receptor-positivity and show distinct chromosomal changes [17, 18]. The more rapidly progressing ductal tumors had an associated higher level of SIBLING expression. OPN was recently identified by microarray analysis as a discriminating marker between ductal and lobular cancer [19]. In our current study, OPN, as well as BSP, DMP1 and DSPP were significantly different between lobular and ductal tumors. SIBLING expression correlated with tumor stages associated with changing size and lymph node involvement (TNM score). These observations are consistent with SIBLING expression coupled with MMP activity modulation having an effect on early tumor progression.

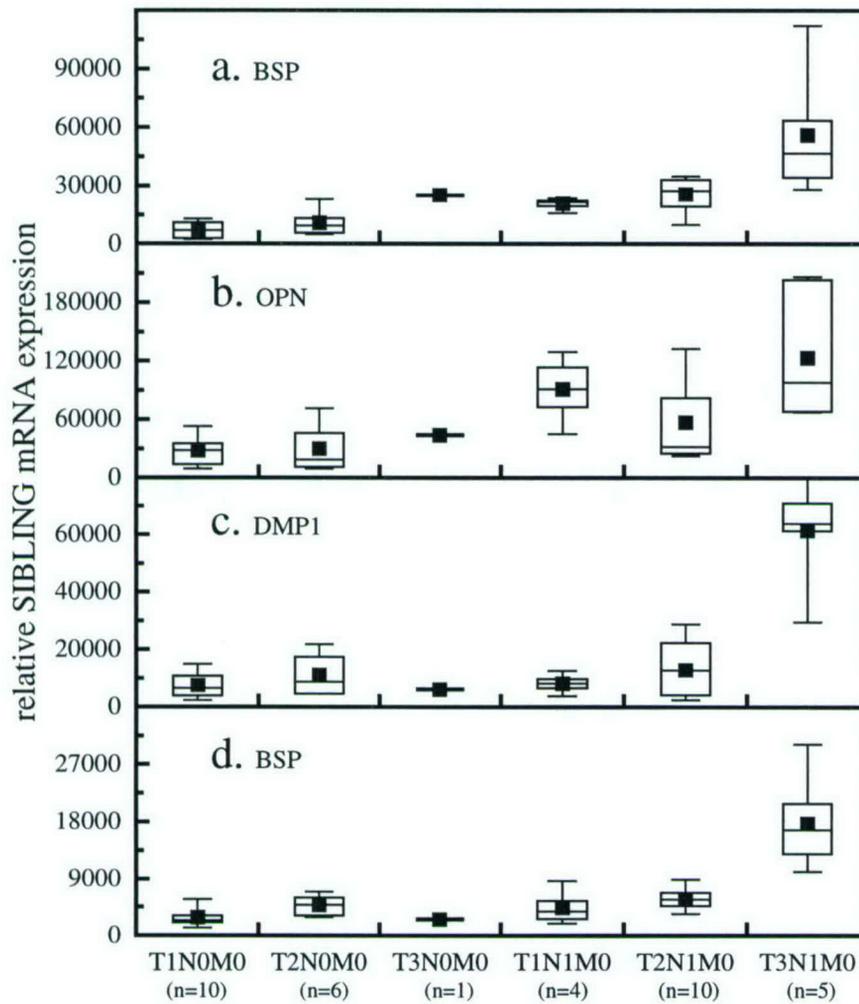


Figure 4. SIBLING levels and TNM stage in breast cancer. The expression values of BSP (a), OPN (b), DMP1 (c), and DSPP (d) by breast cancer tumors were stratified by TNM stage. Top, bottom and line through the middle correspond to 75th percentile, 25th percentile and 50th percentile (median), respectively. Error bar whiskers represent the 10th and 90th percentile, while the solid square symbol indicates the arithmetic mean. For breast cancer TNM staging: T1, tumor \leq 2 cm in greatest dimension; T2, 2 cm < tumor < 5 cm; T3, tumor > 5 cm; N0, no regional lymph node metastasis; N1, metastasis to movable ipsilateral axillary lymph node(s); M0, no distant metastasis. The number of subjects (n) for each group is shown at the bottom

The association between SIBLING expression and tumor TNM stage was tested using Spearman rank correlation and correcting for ties. For BSP Spearman $r = 0.8242$ and p value < 0.0001; for OPN Spearman $r = 0.5098$ and $p = 0.0015$; for DMP1 Spearman $r = 0.4615$ and $p = 0.006$; for DSPP Spearman $r = 0.662$ and $p < 0.0001$. Because of the relatively small number of samples in the various TNM stages, a larger cohort of samples will need to be analyzed for the statistical significance to be realistically assessed. When the analysis of the breast cancer serum samples is completed (expected by the fall of 2004), the sample results will be similarly segregated by TNM stage and the association of serum levels of SIBLINGS with cancer stage determined.

E. Epithelial-Mesenchymal Transition.

A key cancer differentiation event where epithelial cells progressively acquire characteristics of mesenchymal or fibroblasts-like cells has been termed the 'epithelial-mesenchymal transition' [20]. A number of sentinel changes have been identified during the transition, including levels and activity of proteins such as E-cadherin [20], MMP-2 and -9 [21], growth factors and transcription factors [22-26]. The basic leucine zipper-containing transcription factor, TWIST, has been implicated in regulating this transition in breast tumors [27]. In human breast cancers, high level of Twist expression was correlated with invasive lobular carcinoma [27]. Runx2 (OSF2/Cbfa1/AML3) is a homolog of Runt proteins, a group of transcription factors conserved from *C. elegans* to humans that share a 128 amino acid DNA-binding domain termed the Runt domain [28]. RunX2 expression has been associated with tumor progression [23, 29-32]. RunX2 has also been associated with regulating BSP expression [31, 32].

We have used our breast cancer cDNA microarray to investigate RunX2 expression and test for associations with SIBLING and MMP levels. As shown in Figure 5 of Appendix III, the expression of OPN, BSP, DMP1 and DSPP were significantly different between lobular and ductal tumors, with ductal tumors exhibiting higher levels on average. RunX2 expression levels were determined on microarray samples and segregated by tumor type (Figure 5). In contrast to Twist, high levels of RunX2 were associated with ductal tumors.

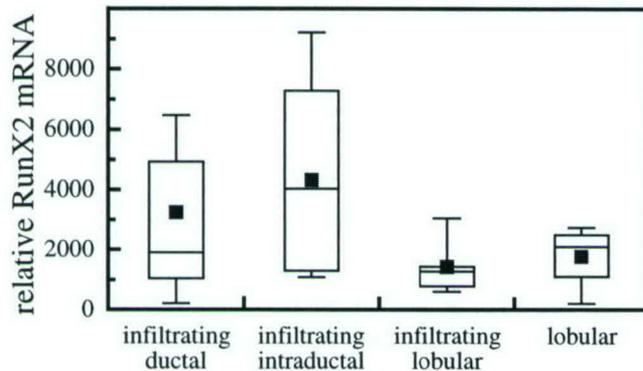


Figure 5. RunX2 expression in breast cancer. The expression values of RunX2 by breast cancer tumors were analyzed using microarray methodology (see Appendix III). Values were grouped by diagnosed tumor type (ductal versus lobular) and the groups compared by box plots. Top, bottom and line through the middle correspond to 75th percentile, 25th percentile and 50th percentile (median), respectively. Error bar whiskers represent the 10th and 90th percentile, while the solid square symbol indicates the arithmetic mean.

The association of the transcription factor RunX2 with SIBLING and MMP expression was also investigated by microarray analysis of breast cancer samples. We have shown that BSP levels were significantly correlated with MMP-2 levels (as reported in Appendix III, figure 4a). MT1-MMP is a membrane bound MMP thought to be involved in activation proMMP-2 [33]. Thus, BSP, MMP-2 and MT1-MMP have the potential for coordinate regulation. RunX2 levels were significantly correlated with MT1-MMP, MMP-2 and BSP levels (Figure 6).

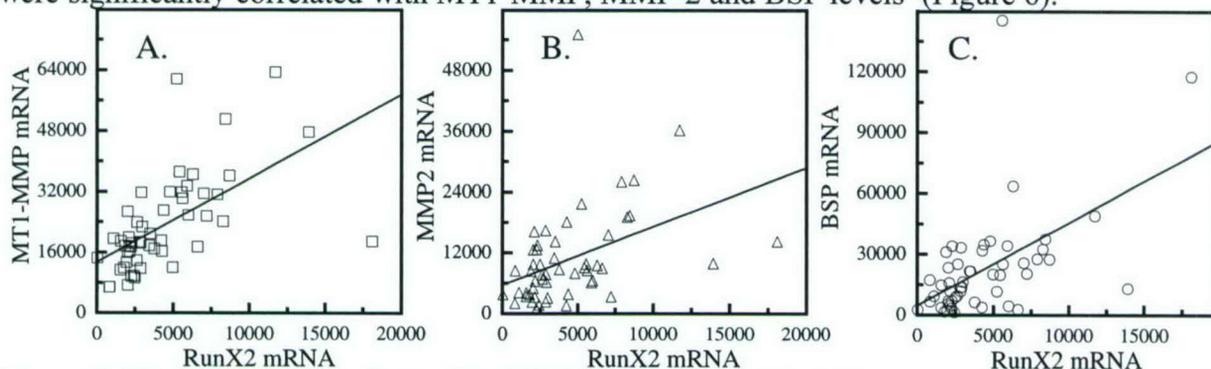


Figure 6. The transcription factor RunX2 is correlated with BSP, MMP-2 and MT1-MMP expression in breast cancer. The expression levels of RunX2, MT1-MMP, MMP-2 and BSP by breast cancer tumors were analyzed using microarray methodology (see Appendix III). BSP and MMP probes were as described in Appendix III. The RunX2 probe (~ 325 bp) was the portion of human RunX2 exon 7 lacking the runt domain. It was subcloned into a PCR vector so no restriction enzyme sites are included. Oligos used were: forward: CCCAAAGCCAGAGTG-GACC, and reverse: GATACGTGTGGGATGTGGC. Probe reactivity quantified by PhosphoImager and ImageQuant software was analyzed by regression analysis. The correlations observed were, for RunX2 and MT1-MMP, $r^2 = 0.35$, $p < 0.0001$; RunX2 and MMP-2, $r^2 = 0.27$, $p < 0.005$; and for RunX2 and BSP, $r^2 = 0.287$ and $p < 0.0001$.

F. Sandwich ELISA development.

During the current reporting period we have developed a sandwich-based ELISA assay for DSPP (Figure 7). Thus, we currently have sandwich ELISAs for BSP, OPN, DMP1 and DSPP for comparison of results with the competitive assays.

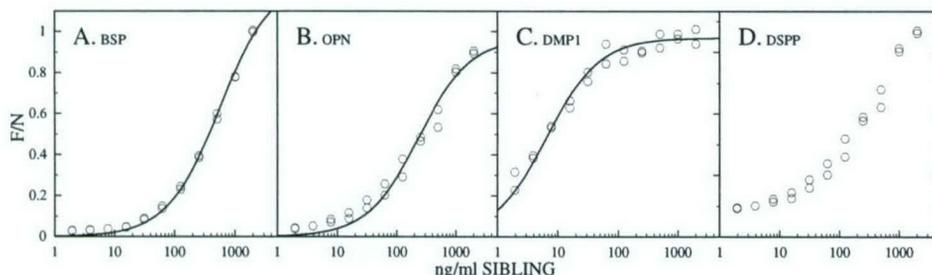


Figure 7. SIBLING indirect sandwich ELISAs. (A) For BSP, the conditions of the assay were coating with capture antibody at 1:1000 (LF-Mab 15, monoclonal) and

second detection antibody (LF 100, polyclonal) at 1:200,000, with a third antibody of anti-species conjugate (horseradish peroxidase) at 1:2,000 and developed for 20 minutes. (B) For OPN, the conditions of the assay were coating with capture antibody at 1:1000 (LF-Mab 15, monoclonal) and second detection antibody (LF 100, polyclonal) at 1:200,000, with a third antibody of anti-species conjugate (horseradish peroxidase) at 1:2,000 and developed for 20 minutes. (C) For DMP1, the conditions of the assay were coating with capture antibody at 1:2000 (LF-Mab 31, monoclonal) and second detection antibody (LF 148, polyclonal) at 1:20,000, with a third antibody of anti-species conjugate (horseradish peroxidase) at 1:2,000 and developed for 20 minutes. For DSPP, the coating with capture antibody at 1:5000 (LF-Mab 21, monoclonal) and second detection antibody (LF 154, polyclonal) at 1:20,000, with a third antibody of anti-species conjugate (horseradish peroxidase) at 1:2,000.

Key Research Accomplishments

Assay Development:

Competitive ELISAs have been developed for 5 different SIBLING family proteins.

Indirect sandwich-based ELISAs have been developed for 4 different SIBLING family proteins.

Assay Application:

The normal distribution pattern of BSP, OPN, DMP1, DSPP and MEPE has been characterized.

The distribution of serum BSP, OPN, DMP1 and DSPP in breast cancer has been described.

In an initial analysis, SIBLING expression levels increase with changing tumor (T) stage.

In contrast to other SIBLINGs, MEPE serum levels are:

- not elevated in breast cancer;
- age-dependent decrease in levels;
- have an inverse correlation with parathyroid hormone levels;
- have a significant positive correlation with total hip and neck BMD;

SIBLING Biology:

SIBLINGs bind to and modulate specific matrix metalloproteinases.

The transcription factor RunX2 is more highly expressed in ductal tumors.

The expression levels of the transcription factor RunX2 were found to be correlated with BSP, MMP-2, and MT1-MMP expression.

Reportable Outcomes

Publications:

Fedarko, N.S., Jain, A., Bianco, P., Ogbureke, K.U.E., Karadag, A., and L.W. Fisher. (2004) Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. *FASEB J.* 18:734-736.

Appendix II. Jain, A., Fedarko, N.S., Collins, M.T., Gelman, R., Ankrom, M.A., Tayback, M.,

and L.W. Fisher. (2004) Serum levels of matrix extracellular phosphoglycoprotein (MEPE) in normal humans correlate with serum phosphorus, parathyroid hormone and bone mineral density. *J. Clin. Endo. Metab.* In Press.

Manuscripts

Fisher, L.W., Jain, A., Tayback, M., and N.S. Fedarko. (2004) Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) gene family expression in different cancers. *Clin. Cancer Res.* Submitted.

Conclusions

Summary. The development of competitive ELISAs and sandwich-based ELISAs to measure serum levels of SIBLING gene family members has been a necessary requirement in order to evaluate the utility of these potential markers in breast cancer detection. Initial results in applying these assays describe a gaussian distribution of SIBLINGs (with the exception of MEPE) in the serum of normal individuals. In serum from subjects with cancer, serum SIBLING levels were increased (for BSP, OPN, DMP1 and DSPP) in the presence of disease. In breast cancer, in particular, BSP and OPN display greater sensitivity and specificity than DSPP. More normal and breast cancer sera need to be analyzed for ROC analysis of DMP1 sensitivity and specificity. All of our data so far is consistent with SIBLINGs behaving as early markers of breast cancer progression. This conclusion is reached by noting that (a) increased expression levels are not associated with metastases, (b) increased expression levels are observed with increasing tumor size (T status in TNM staging) and lymph node involvement (N status in TNM staging) (c) SIBLINGs can act biologically to modulate matrix metalloproteinase (MMP) activity. MMP activation is required for early tumor progression (remodeling the extracellular matrix scaffolding to yield space for growth and for angiogenesis). The ability of SIBLING levels to distinguish between lobular versus ductal breast carcinomas could be reflecting the differences in multifocality between the two types of breast cancer or it may be an indication of differences in rate of progression between the two classifications. The transcription factor RunX2 was found to be significantly elevated in ductal tumors when compared with lobular tumors and the levels of RunX2 were correlated with BSP, BSP's cognate ligand MMP-2, and with MT1-MMP. This pattern of gene expression may be reflecting a specific type of "mesenchymal-like" phenotype that is associated with ductal tumors.

Future plans.

As an Associate Member of the NCI's Early Detection Research Network, the P.I. now has access to Tissue arrays (<http://www3.cancer.gov/prevention/cbrg/edrn/associate.html>). The breast cancer array consists of 2039 total cores, with a breakdown by core histology of 600 invasive tumor, 421 in situ tumor, 152 metastatic lesions, 70 atypical hyperplasia, 69 ductal hyperplasia, 548 normal matched tissues, 51 benign tissues, 95 normal from normal cases, and 33 other. At a future date, we would like to use our antibodies against SIBLINGs to determine the levels and localization of SIBLINGs in these subtypes of breast cancer.

What we will have learned. There currently does not exist a serum marker that can be used to detect breast cancer at an early stage. Because of evolving knowledge concerning both the biological actions of SIBLINGs (modulating MMPs) and their serum levels and tumor tissue expression, members of the SIBLING gene family hold promise as biomarkers for early cancer detection. The completion of the research described in this progress report and in the original grant application will enable the utility of these breast cancer biomarkers to be defined. Once completed, the groundwork will be laid for subsequent clinical trials of these biomarkers. A further byproduct of the research conducted so far, is an expansion of our understanding of the basic biology involved in tumor progression, identification of novel alternative methods of MMP activation and of potential pathways for therapeutic intervention.

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Appendices

Appendix I. Fedarko, N.S., Jain, A., Bianco, P., Ogbureke, K.U.E., Karadag, A., and L.W. Fisher. (2004) Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. FASEB J. 18:734-736.

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Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases¹

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SPECIFIC AIMS

Objectives were to identify proteins that co-purified with members of the small integrin-binding ligand N-linked glycoprotein (SIBLING) gene family and characterize structural and functional consequences of their binding interactions.

PRINCIPAL FINDINGS

1. Specific MMPs co-purify with SIBLINGs

Individual SIBLING family members BSP, OPN and DMP1 were subcloned into an adenovirus system and expressed in human bone marrow stromal cells. Each SIBLING was purified from the serum-free media to $\geq 95\%$ purity by anion exchange chromatography under non-denaturing conditions. When purity was assessed by zymography, each HPLC purified SIBLING exhibited a single band of proteolytic activity. Bands originally visible on the zymogram did not appear in gels treated with 1,10-phenanthroline, showing that co-purifying proteolytic activity arose from metalloproteinases. Identity of proteolytic bands was determined by Western blotting and probing with specific antibodies against MMPs. MMP-2 co-purified with BSP, MMP-3 with OPN and MMP-9 with DMP1.

Specificity observed was confirmed by showing that purified SIBLINGs could be used to affinity purify their respective MMPs from conditioned media containing several different MMPs. When aliquots of eluted fractions were analyzed by zymography, positive bands for multiple MMPs were visible in the flow through peak. Fractions that eluted at ~ 0.3 M salt were analyzed by Western blot and immunoreactive MMP-2 (from the BSP affinity column) and immunoreactive MMP-3 (from the OPN affinity column) were identified. A DMP1 affinity column was not made due to insufficient amounts of highly purified DMP1.

2. SIBLING and MMP binding specificity

Co-purification from similar media of a single but different MMP with each SIBLING demonstrated that

specific interactions were occurring between the proteins. Binding interactions between recombinant MMP-2, MMP-3 and MMP-9 and purified recombinant SIBLINGs were investigated. Relative abundance of tryptophan residues in the MMPs was exploited by carrying out intrinsic fluorescence studies of purified, authentic MMP protein binding to SIBLINGs. Titration of proMMP-2 with BSP yielded a quenching of the MMP's tryptophan emission spectra and a saturable binding curve. Addition of OPN to proMMP-3 and DMP1 to proMMP-9 also yielded fluorescent signal quenching and saturable binding functions. Stoichiometry of binding between SIBLINGs and their respective proMMPs was 1:1. Scatchard analysis indicated binding constants in the nM range. Quenching of the tryptophan fluorescent signal is consistent with a gross conformational change as a result of binding. Fluorescent binding studies were also carried out using mixed pairs of SIBLINGs and pro- and active-MMPs. SIBLINGs and MMPs showed consistent specificity in their partnering, with BSP binding to pro- and active MMP-2, OPN with pro- and active MMP-3, and DMP1 with pro- and active-MMP-9. Other combinations of SIBLINGs and MMP's exhibited either no saturable binding or binding that was orders of magnitude weaker.

3. SIBLING-MMP complexes modify the protease activity

Fluorescence spectroscopy observations suggesting that SIBLING binding induces conformational changes in their corresponding MMP partner led to an investigation of whether SIBLING binding affected MMP structure. Addition of SIBLING to pro-MMP did not appear to promote autocatalysis to the active form. An increased susceptibility of SIBLING-proMMP complexes to cleavage and activation by plasmin (a protease that is normally an inefficient activator) was seen and was

¹ To read the full text of this article, go to <http://www.fasebj.org/cgi/doi/10.1096/fj.03-0966fje>; doi: 10.1096/fj.03-0966fje

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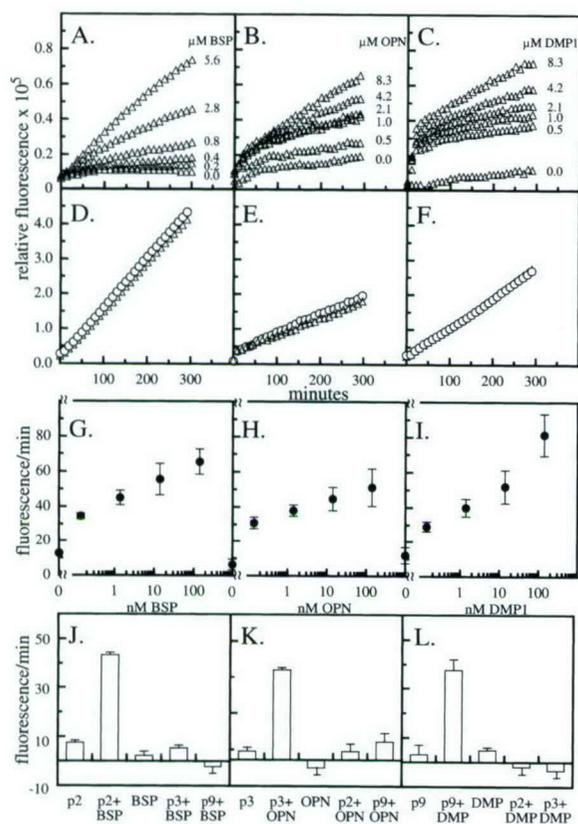


Figure 1. Modulation of MMP activity by SIBLINGs. Protease activity was followed by incubating fluorescent substrate with 1.4 nM proMMP-2 (A), proMMP-3 (B), and proMMP-9 (C) and increasing concentrations of BSP, OPN, or DMP1, respectively. Activity of equimolar concentrations (1.4 nM) of active MMP-2 \pm BSP (D), MMP-3 \pm OPN (E), and MMP-9 \pm DMP1 (F) in the same assay showed no difference. The same assay was also employed to determine a low SIBLING concentration dose response in activity of proMMP-2 + BSP (G), proMMP-3 + OPN (H), and proMMP-9 + DMP1 (I). Activity of 1.4 nM BSP (J), OPN (K) and DMP1 (L) with correctly matched proMMPs and mismatched proMMPs as well as of proMMPs or SIBLINGs alone were analyzed by determining change in fluorescence/minute over the first 3 h of incubation. Values plotted represent the mean of three combined experiments \pm SD.

consistent with SIBLING binding altering proMMP structure.

To measure potential biological effects, a fluorescent substrate assay was employed to screen SIBLING modulation of proMMP activity. Pro-MMP-2, -3, and -9 were reacted in combination with increasing concentrations of SIBLINGs (either BSP, OPN, or DMP1) and enzyme activity was measured by increased fluorescence signal. Increased proteolytic activity was observed for all three strong proMMP+SIBLING binding pairs (Fig. 1A–C). When the strong binding SIBLING was added to its corresponding active MMP, enzymatic activity was not significantly changed indicating that binding of the SIBLING with its active MMP partner did not interfere with normal proteolytic activity (Fig. 1D–F). Properly matched SIBLING-proMMP pairs showed a dose-response increase in the rate of substrate digestion (Fig.

1G, H, I). Incubation of SIBLING alone with substrate was no different from pro-MMP alone or mismatched SIBLING MMP pairs, showing the increase in activity in the proMMP + SIBLING was not caused by any residual proteolytic activity that co-purified with the SIBLING (Fig. 1J–L). Given that there was no observed increase in the amount of propeptide-free enzyme in all of these SIBLING-proMMP pairs, it is reasonable to hypothesize that the increase in activity is due to a conformational change in the protease which allows its propeptide to be removed from the active site and thereby permit digestion of both small and large macromolecular substrates.

4. SIBLINGs restore activity to inhibited MMPs

Quenching of tryptophan fluorescence and increase in activity caused by SIBLING binding to proMMP is consistent with an alteration in the local structure near the active site. The effect of SIBLINGs on the ability of small molecular weight inhibitors to modulate MMP activity was investigated next. SIBLINGs were able to increase proMMP activity in the presence of specific small molecular weight inhibitors of MMPs, but not in the presence of 1,10 phenanthroline (which disrupts MMP activity by chelating and removing the active site required zinc ion). Active forms of MMPs also exhibited quenching of tryptophan fluorescence emission upon binding their specific SIBLING partner. The possibility that SIBLING binding also altered inhibitor interaction with active MMPs was investigated. Specific low molecular weight inhibitors were used to block active MMP activity. Addition of the corresponding SIBLING, however, rescued much of the original activity even in presence of equimolar amounts of specific inhibitor. As was the case for proMMPs, SIBLINGs were not able to restore activity to active MMPs treated with 1,10 phenanthroline. When the complex of equimolar active MMP + SIBLING was treated with increasing concentrations of the inhibitor, significant loss of activity was observed but only at substantially higher concentrations.

Because MMPs occur *in vivo* associated with inhibitors (tissue inhibitors of matrix metalloproteinases, TIMPs), the effect of SIBLINGs on the activity of MMP + TIMP complexes was also investigated. As expected, presence of TIMPs reduced the enzymatic activity of propeptide-free, active MMP. Addition of the correctly matched SIBLING to active MMP + TIMP complex caused a restoration of proteolytic activity. It is a reasonable hypothesis that conformational change in the active MMP upon binding its SIBLING partner lowers affinity of the TIMP (and low molecular weight inhibitors) for the active site of the MMP thereby enabling substrate access.

5. Reversal of SIBLING-induced activity by factor H

BSP, OPN and DMP1 have previously been shown to bind to factor H with high affinity, 10–100-fold higher than that just described for their partner MMPs. Gela-

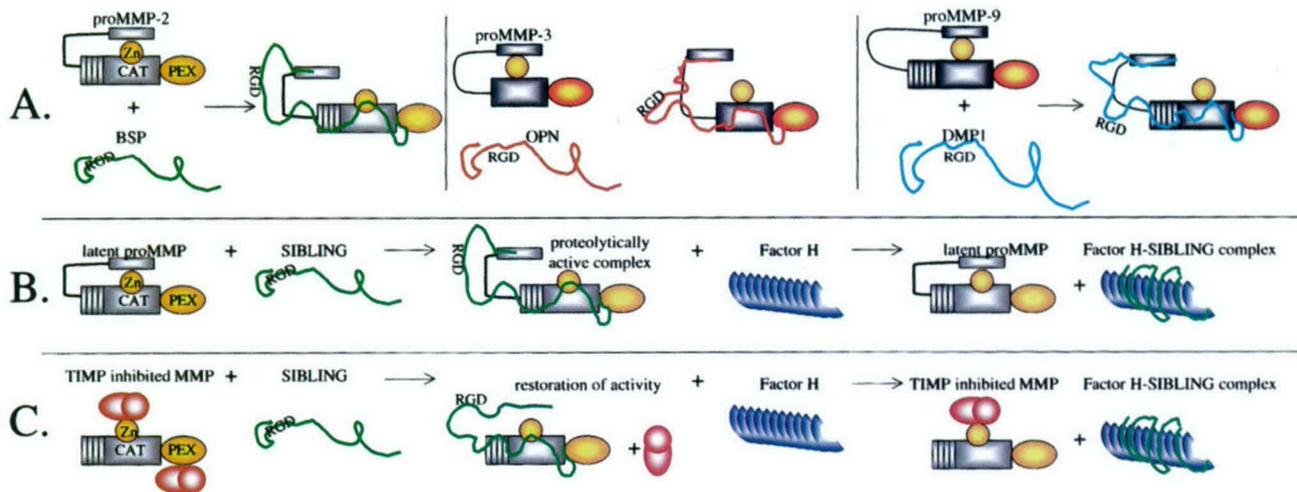


Figure 2. Schematic diagram of SIBLING-MMP interactions. *A*) Specificity of binding and activation was observed for BSP and proMMP-2, OPN and proMMP-3, and DMP1 and proMMP-9. *B*) SIBLING binding to a specific proMMP results in increased proteolytic activity in absence of propeptide cleavage. *C*) SIBLING binding to TIMP inhibited MMP leads to restoration of activity. In both cases, complement factor H, with its higher affinity for SIBLINGs may strip the SIBLING from the complex and proteolytic activity is lost.

tin and casein fluorescein conjugate assays were used to investigate whether factor H can compete with MMPs for SIBLING binding and thereby affect each SIBLING's interactions with its respective proMMP and active MMPs. TIMP-inhibited MMPs which had regained enzymatic activity by the addition of their corresponding SIBLING were treated with factor H and a significant reduction in the SIBLING-induced recovered activity for MMPs was observed. Higher affinity of factor H for SIBLING protein appears to promote removal of SIBLING from the SIBLING-MMP complex thereby permitting MMP to reverse its conformation and allow TIMP to again bind to the active site and re-inhibit the enzyme. The action of factor H on the SIBLING-mediated activation of proMMPs was also investigated. Addition of factor H caused the rate of substrate digestion by SIBLING-activated proMMP complex to decrease suggesting that removal of SIBLING from proMMP resulted in re-inactivation of catalytic activity by still-attached propeptide. These results support the hypothesis that propeptide is not removed in order to create enzymatic activity in proMMP-SIBLING pairs.

CONCLUSIONS AND SIGNIFICANCE

Results describe a novel, alternative method of MMP modulation (**Fig. 2**). SIBLING binding was associated with activation of latent pro-MMPs though this

activation did not require cleavage of the propeptide. However, SIBLING binding did increase susceptibility of the propeptide to cleavage by plasmin. SIBLINGs and MMPs showed specificity in their partnering, with BSP binding to and "activating" proMMP-2, OPN with proMMP-3, and DMP1 with proMMP-9. Restoration of activity to TIMP-inhibited MMPs upon SIBLING binding demonstrates that even in presence of TIMPs, MMPs may be enzymatically active in regions of locally high concentrations of specific SIBLINGs. The observation that complement factor H can compete with MMPs for SIBLINGs and successfully strip the SIBLING from the MMP complex suggests that activation of proMMP or reactivation of TIMP-inhibited MMPs by simple binding of their respective SIBLINGs will be limited to short distances from their sites of secretion due to abundance of factor H in the body.

SIBLING expression has been correlated with cancer progression and severity and it is interesting to consider that these proteins may be locally activating their corresponding proteases *in vivo*. From a clinical standpoint, SIBLINGs were found to restore activity to propeptide-free MMPs whose activity had been blocked by both natural and synthetic inhibitors. SIBLINGs are induced by neoplasms *in vivo* and their modulation of MMP activity might contribute to the relative lack of efficacy seen in recent clinical trials of MMP inhibitors **[F]**

Three small integrin-binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases

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ABSTRACT

Matrix metalloproteinases (MMPs) are critical for development, wound healing, and for the progression of cancer. It is generally accepted that MMPs are secreted in a latent form (proMMP) and are activated only upon removal of their inhibitory propeptides. This report shows that three members of the SIBLING (Small, Integrin-Binding Ligand, N-linked Glycoprotein) family can specifically bind ($K_d \approx nM$) and activate three different MMPs. Binding of SIBLING to their corresponding proMMPs is associated with structural changes as indicated by quenching of intrinsic tryptophan fluorescence, increased susceptibility to plasmin cleavage, and decreased inhibition by specific natural and synthetic inhibitors. Activation includes both making the proMMPs enzymatically active and the reactivation of the TIMP (tissue inhibitors of MMP) inhibited MMPs. Bone sialoprotein specifically binds proMMP-2 and active MMP-2, while osteopontin binds proMMP-3 and active MMP-3, and dentin matrix protein-1 binds proMMP-9 and active MMP-9. Both pro and active MMP-SIBLING complexes are disrupted by the abundant serum protein, complement Factor H, thereby probably limiting SIBLING-mediated activation to regions immediately adjacent to sites of secretion in vivo. These data suggest that the SIBLING family offers an alternative method of controlling the activity of at least three MMPs.

Key words: bone sialoprotein • osteopontin • dentin matrix protein 1

Members of the SIBLING family contain the integrin binding tripeptide, Arg-Gly-Asp (RGD) as well as several conserved phosphorylation and N-glycosylation sites (1). All of the genes cluster on human chromosome 4 and are expressed in the skeleton. There are five known members of the SIBLING family: bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE) (1). Normally BSP is produced by osteoblasts, osteoclasts, osteocytes, and hypertrophic chondrocytes (2) and through its Arg-Gly-Asp sequence binds to the integrin, $\alpha_v\beta_3$. OPN (also known as Secreted Phosphoprotein 1, 2ar, and early T-lymphocyte activation 1) is expressed in multiple tissue types and can bind to $\alpha_v\beta_3$ (3–5) as well as CD44 (6,

7). DMP1 expression has been found in teeth and bone (8, 9) and can also interact with both $\alpha_v\beta_3$ and CD44 (10). OPN, BSP and DMP1 can protect cells from complement attack via initial binding to $\alpha_v\beta_3$ integrin (all three) or CD44 (OPN and DMP1) on the cell surface. The SIBLING then binds complement Factor H and the membrane-bound complex acts as a cofactor to complement Factor I thereby quenching complement-mediated cell lysis (10, 11).

Recent observations using paraffin sections have shown that BSP and OPN are expressed by multiple malignant tissues, including breast (12–14), prostate (15, 16), lung (17), and thyroid (18, 19) cancers. BSP expression is associated with poor survival in breast cancer (13) and prostate cancer (15). Similarly, OPN expression is associated with clinical severity in lung cancer (20), lymph node negative breast cancer (14), gastric cancer (6), and perhaps ovarian carcinoma (21). Recently, DMP1 has been shown to be strongly up-regulated in lung cancer (22). The neoplastic expression pattern of other SIBLING members has not been defined. Recently, OPN and/or BSP were found to be elevated in the blood of breast, lung, colon, and prostate cancer patients (23).

Matrix metalloproteinases (MMPs) are a class of hydrolytic enzymes defined by a common structure and a requirement for zinc in the active site. Currently, it is accepted that the inhibitory propeptide must be removed before the MMP can be enzymatically active. After the removal of the propeptide, the MMP typically remains active until a TIMP (tissue inhibitor of matrix metalloproteinase) binds and inactivates the protease. In addition to their roles in tissue remodeling, MMPs have been postulated to play major roles in tumor cell progression and metastasis (24). In this report, we show that at least three members of the SIBLING family specifically bind to different proMMPs resulting in conformational changes that are catalytically active. Furthermore, it is shown that active MMPs inhibited by either TIMPs or low-molecular-weight synthetic inhibitors are reactivated by their corresponding SIBLING and that all these activations can be essentially reversed by complement Factor H.

METHODS

Reagents

ProMMPs, their active counterparts, TIMP1, and monoclonal antibodies that recognize both latent and active forms of MMP-2, MMP-3, and MMP-9 were obtained from Oncogene Research Products (Boston, MA) and Research Diagnostic Systems, Inc. (Minneapolis, MN). Purified human Factor H protein was obtained from Quidel Corporation (San Diego, CA). ToyoPearl activated AF-Tresyl-650 M and TSK QAE resins were obtained from TosoHaas, Inc. (Montgomeryville, PA). The MMP-2 Inhibitor I, *cis*-9-octadecenoyl-N-hydroxylamide; MMP-3 Inhibitor II, N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid; MMP-9 Inhibitor I, N-hydroxy-1-(4-methoxyphenyl)sulfonyl-4-(4-biphenylcarbonyl)piperazine-2-carboxamide, and plasmin were obtained from Calbiochem (La Jolla, CA). The small molecular weight fluorescently quenched substrates MET-05, (7-methylcoumarin-4-yl acetic acid)-Arg-Pro-Lys-Pro-Val-Glu-Ape-Trp-Arg-Lys-(dinitrophenyl)-NH₂; MET-08, (7-methylcoumarin-4-yl acetic acid)-Arg-Pro-Lys-Pro-Tyr-Ala-Ape-Trp-Met-Lys-(dinitrophenyl)-NH₂; and MET-09, (7-methylcoumarin-4-yl acetic acid)-Pro-Leu-Gly-Leu-(diaminopropionic acid)-Ala-Arg-NH₂ were purchased from Enzyme System Products (Livermore, CA). TIMP2 was a generous gift of Dr. H. Birkedal-Hansen, NIDCR, NIH.

SIBLING production and purification

Recombinant human BSP, human OPN and bovine DMP1 with good post-translational modifications were expressed in eukaryotic cells using adenovirus constructs as described previously (10, 11). Recombinant SIBLINGs were purified from the serum-free media of the same primary cell cultures infected with their respective viruses by anion exchange chromatography under nondenaturing conditions (11). For binding studies as well as MMP activation assays, SIBLINGs were further purified by treatment with a chaotropic buffer (4 M guanidine HCl in 40 mM Tris, 1 mM DTT, pH 7.4) for 15 min at room temperature followed by dialysis and lyophilization. SIBLINGs are flexible in solution and lack sufficient cysteine residues for disulfide bond formation (1). Treatment of the SIBLINGs with denaturants (reducing agents or chaotropic agents) does not affect the "structureless" nature of SIBLINGs but will denature other proteins and enable the isolation of SIBLINGs that are free of copurifying MMPs.

SIBLING affinity chromatography

SIBLING affinity columns were made by conjugating BSP or OPN to ToyoPearl AF-Tresyl-650 M resin. 3.3 mg BSP was reacted with 0.33 g Tresyl resin in 50 mM Tris pH 8.0 containing 0.5 M NaCl overnight at 4°C. Resin was rinsed twice with 20 ml Tris buffer, and any remaining active groups were blocked by incubation for 2 h at room temperature in Tris buffer. Conjugated resin was packed into a 10 x 0.66 cm Omnifit glass column (Rainin, Woburn, MA) and equilibrated in 20 mM Tris-HCl buffer, pH 7.4 containing 10 mM CaCl₂, 0.02% NaN₃, 0.05% Brij 35, and 12 mM EDTA. For OPN, the affinity column consisting of 4.5 mg OPN coupled to 0.4 g of Tresyl resin following the same steps as for the BSP affinity column. Serum-free conditioned medium (B16F10 melanoma cell line) was injected onto the columns and washed in the equilibration buffer until the UV absorbance at 280 nm returned to baseline. A linear gradient to 1.0 M NaCl over 60 min (1.0 ml/min flow rate) was used to elute the bound MMP. Fractions were analyzed by zymography as below.

SDS PAGE, zymography

10% zymogram gelatin and 12% zymogram casein gels were obtained from Invitrogen, Inc. (Carlsbad, CA). Samples in zymogram gel sample buffer were electrophoresed at a constant 125 V for 90 min. Gels were processed for zymography according to the manufacturer's instructions, stained with 0.5% Coomassie Blue R 250, and bands were visualized by dynamic integrated exposure (integrating a 1/30 s exposure over 3 s) using an EagleEye II imaging system (Stratagene Corp., La Jolla, CA). For subsequent visualization of the SIBLINGs, gels were completely destained in high methanol destaining solution, rinsed in the 25% isopropanol through 4 changes over a 12 h period, and then reacted with the StainsAll solution (0.1% (w/v) containing 25% (v/v) isopropanol, 5% (v/v) formamide and 15 mM Tris-HCl, pH 8.8). 12% acrylamide gels were employed to parallel SIBLING and MMP zymogram profiles and the proteins transferred to nitrocellulose for analysis by Western blot.

Western blotting

Samples diluted in gel sample buffer were resolved by Tris/glycine SDS 12% polyacrylamide gels (Invitrogen, Inc., Carlsbad, CA) and transferred to nitrocellulose following standard

conditions (25). Nitrocellulose membranes were rinsed with Tris-buffered saline (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.05% Tween 20 (TBS-Tween). After 1 hour of incubation in blocking solution (TBS-Tween + 5% nonfat powdered milk) at room temperature, 1 μ g/ml primary antibody (monoclonal anti-MMP) was incubated overnight at 4°C. The blot was washed in TBS-Tween four times for 5 min with TBS-Tween and then HRP-conjugated goat anti-mouse IgG (100 ng/ml) in TBS-Tween + 5% milk was added and incubated for 2 h at room temperature. After washing, enhanced chemiluminescence reagents were employed for signal detection (Pierce Chemical Co., Chicago, IL) with X-ray film.

Fluorescent binding studies

Intrinsic tryptophan fluorescence was monitored by excitation at 295 nm and emission from 300 to 500 nm using a Photon Technology International Series M fluorimeter. The relative change in fluorescence in the area under the emission curve was used to determine binding curves by calculating fractional acceptor saturation vs. nM SIBLING added. Fractional acceptor saturation (f_a) was determined by calculating $f_a = (y - y_f)/(y_b - y_f)$, where y_f and y_b are the area under the curve of the fluorescent emission profile of free and fully bound MMP, respectively. Scatchard plots were made by fitting the transformed data to the function $r/[C_S] = n/K_d - r/K_d$, where r represents the binding function, C_S represents total ligand concentration, n represents the number of binding sites, and K_d represents the dissociation constant. The initial latent or active MMP concentration was 3.5 nM and concentrated SIBLINGs were added in nM amounts. Both latent and active MMPs, as well as SIBLINGs were dissolved in Hank's balanced salt solution. To determine stoichiometry, a titration was carried out under conditions with an excess of MMP (10-fold higher than the dissociation constant). The stoichiometry was ascertained in each case by transforming the data into a plot of fractional saturation vs. the ratio of total ligand C_S to total acceptor C_A . Extrapolating the two linear segments of the graph to their point of intersection defined the MMP and SIBLING stoichiometry.

High-molecular-weight substrates

Fluorescein-conjugated gelatin (Molecular Probes, Inc., Eugene, OR) or casein (Pierce Chemical Co., Chicago, IL) substrates were used to follow proteolytic activity on more natural macromolecular substrates. These substrates are so highly substituted with fluorescein moieties that the fluorescent signal is self-quenched until proteolytic cleavage liberates fragments, and a robust fluorescent emission is measured. 1.4 nM MMP (latent or active MMP-2, MMP-3, and MMP-9) was reacted with increasing concentrations of SIBLINGs or buffer alone and 12.5 μ g/ml of the fluorescein-substrate conjugate in 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM CaCl₂. Fluorescent data were acquired with excitation at 485 nm and emission at 535 nm. In indicated experiments, the general MMP inhibitor 1,10 phenanthroline was added. A final 1,10 phenanthroline concentration of 1 mM in zymograms and 1.4 μ M in enzyme assays were made by the appropriate dilution from a 100 mM stock solution dissolved in DMSO. In experiments with TIMPs, the inhibitors were preincubated in equimolar amounts with the MMPs before the addition of the SIBLINGs. Factor H was added at levels equimolar to the SIBLINGs.

Low-molecular-weight substrates

The activities of latent and active MMPs in the presence and absence of SIBLINGs and after plasmin treatment were measured using the small fluorescence-quenched substrates (26). These

substrates have two fluorescent moieties covalently attached usually at either end of the peptide such that the fluorescent signal of the intact substrate is effectively quenched. Cleavage of the peptide results in loss of quenching and an enhanced fluorescence signal. The peptide substrates were initially dissolved in DMSO and each assay carried out at 25°C in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.01% Brij-35 and 1% DMSO (v/v). ProMMPs (140 nM) were added to 400 nM substrate in the presence or absence of SIBLING (140 nM). Substrate cleavage was monitored using a Perkin Elmer Victor 2 multilabel plate reader with 330 excitation and 390 emission filters.

Plasmin susceptibility

SIBLING and proMMP concentrations were 140 nM, plasmin 20 nM in 50 mM HEPES, pH 7.5, 150 mM NaCl, 8 mM CaCl₂, 0.01% Brij 35, and 1% DMSO. Low-molecular-weight fluorescently quenched methylcoumarin-derivatized substrates were employed at a concentration of 400 nM to follow MMP activity in the presence and absence of SIBLINGs and plasmin. Plasmin itself will not digest the peptide substrate under these conditions.

RESULTS

SIBLINGs and MMPs copurify

Full-length BSP, OPN, and DMP1 cDNA were subcloned into adenovirus vectors and expressed in human bone marrow stromal cells. Each SIBLING was purified from the serum-free media of the same primary cell cultures infected with their respective viruses to ≥ 95% purity by anion exchange chromatography under nondenaturing conditions (11). Interestingly, when purity was further assessed using casein zymography, each HPLC-purified SIBLING exhibited a single but different band of proteolytic activity (Fig. 1A and B). For DMP1 (*M_r* of 110–120 kDa on PAGE), a zymogram-positive band was seen at ~84 kDa, consistent with MMP-9. BSP (*M_r* of 70–80 kDa) had an associated zymogram-positive band of ~66 kDa, consistent with MMP-2. Finally, OPN (*M_r* of 50–60 kDa) had an associated zymogram positive band of ~45 kDa consistent with MMP-3. Bands originally visible on the zymogram did not appear in gels treated with 1,10-phenanthroline, showing that the copurifying proteolytic activity arose from metalloproteinases (data not shown). This copurification of MMP-2 with BSP, MMP-3 with OPN, and MMP-9 with DMP1 was verified by electrophoresing the same preparations of SIBLINGs on SDS polyacrylamide gels followed by blotting to nitrocellulose and probing with monoclonal antibodies against the three MMPs (Fig. 1C, D, and E).

SIBLING and MMP affinity purification

The specificity observed in copurification of individual MMPs with recombinant SIBLINGs was confirmed in two cases by showing that SIBLINGs could be used to affinity purify their respective MMPs from conditioned media. BSP and OPN were conjugated to resins and serum-free conditioned B16F10 medium was used as a source of MMPs, including MMP-2, MMP-3, and MMP-9. Elution of bound material in a salt gradient yielded multiple peaks as monitored at A 280 nm (Fig. 2A and B). Aliquots of fractions (denoted by arrows) were analyzed by zymography. Zymogram bands with a *M_r* corresponding to latent and active MMP-2 (for the BSP affinity column) and proMMP-3 (for the OPN affinity column) and were visible only in peaks eluted at higher (~0.3 M) salt (Fig. 2C and D). The gradient-eluting bands did not appear

in zymogram gels treated with 1,10-phenanthroline. The same fractions were also analyzed by Western blot and immunoreactive MMP-2 (from the BSP affinity column) and immunoreactive MMP-3 (from the OPN affinity column) were identified (Fig. 2E and F). A DMP1 affinity column was not made due to insufficient amounts of highly purified protein.

SIBLING and MMP binding specificity

Copurification by virtually identical methods of a single but different MMP with each SIBLING suggested that there were specific and relatively strong interactions occurring between the proteins. The relative abundance of tryptophan residues in the MMPs was exploited by carrying out intrinsic fluorescence studies of purified, authentic latent and active MMP protein binding to SIBLINGs. ProMMP-2 contains 15 tryptophan residues, proMMP-3 contains 8, and proMMP-9 contains 13. In contrast, mature OPN and DMP1 each contain 1 tryptophan residue whereas BSP contains none. Intrinsic tryptophan fluorescence measurements of macromolecules yield information about conformation, binding, and solvent interactions. Titration of proMMP-2 with BSP yielded a proportional amount of quenching of the MMP's tryptophan emission spectra (Fig. 3A). Titration of proMMP-3 with OPN and proMMP-9 with DMP1 also yielded a similar quenching of the MMPs' tryptophan emission spectra (Fig. 3B and C). Quenching of the MMP tryptophan fluorescent signal is consistent with a significant conformational change (exposing internal tryptophan residues to a more polar environment) as a direct result of the binding of its corresponding SIBLING. The binding of each SIBLING to its preferred proMMP was saturable (Fig. 3D, E, and F). When the interaction of SIBLINGs with propeptide-free (active) MMPs was studied by intrinsic fluorescence, saturable binding was again observed only for the specific pairs; BSP and active MMP-2, OPN and active MMP-3, and DMP1 and MMP-9 (Fig. 3D, E, and F). K_d values of 2.9 ± 0.9 and 0.3 ± 0.1 nM for BSP binding to latent and active MMP-2; 0.55 ± 0.01 and 0.17 ± 0.01 nM for OPN binding to latent and active MMP-3, and 0.41 ± 0.04 and 0.77 ± 0.01 nM for DMP1 binding to latent and active MMP-9 were determined from Scatchard plots (Fig. 3G, H, and I). The stoichiometry of binding between SIBLINGs and their respective proMMPs was found to be 1:1 for all three combinations (Fig. 3J, K, and L). All combinations of mismatches of SIBLINGs and both pro and active MMPs (ex. BSP with proMMP-3 or DMP1 with MMP-2) yielded either no binding curve at all or no saturation, thereby again showing specificity of the SIBLING to MMP interactions (data not shown).

SIBLING–MMP complexes modify the protease activity

The fluorescence spectroscopy observations suggesting that SIBLINGs induce conformational changes in their corresponding MMP partners led to an investigation of whether SIBLING binding affected MMP structure and function. First, samples of correctly matched proMMPs and SIBLINGs were incubated for 1 h at room temperature and resolved by casein and gelatin zymography to monitor propeptide presence. The addition of SIBLING to proMMP did not appear to cause significant cleavage (auto-activation) to the lower molecular weight, active form (Fig. 4).

Next the SIBLING–proMMP complex was tested for susceptibility of the proMMP to removal of its propeptide by an added protease. Plasmin is normally an inefficient activator of MMP-2 and MMP-9 (27) and treatment of proMMP-2 or proMMP-9 with plasmin resulted in minimal generation of zymogram bands corresponding to the "active," that is, lower M_r forms (Fig. 5A). The addition of BSP to proMMP-2 and DMP1 to proMMP-9 and subsequent incubation with

plasmin, however, did result in greater levels of the small, active form of the MMP as measured by zymography. As controls, neither the SIBLINGs alone, nor SIBLINGs incubated with plasmin led to the appearance of zymogram bands corresponding to active MMPs.

An increased susceptibility of SIBLING–proMMP complexes to cleavage and activation by plasmin was also seen when enzyme activity was monitored by incubation with the MMP-specific, small, fluorescent substrates. Plasmin activation of proMMP-2 and proMMP-9 was increased by treatment with SIBLING (BSP and DMP1, respectively). Surprisingly, treatment of proMMP with SIBLING alone also led to a two- to threefold increase in proteolytic activity, as measured using the low-molecular-weight substrates. Neither BSP nor DMP1 alone exhibited any activity, demonstrating that the purification procedure for SIBLINGs removed residual MMPs that had initially copurified with them, (Fig. 5B). Taken together, these data are consistent with SIBLING binding altering proMMP structure. The SIBLING–proMMP complex displays catalytic activity on small substrates and the propeptide is more readily cleaved by an added protease than is the proMMP alone.

SIBLINGs and proMMP activation

To measure potential enzymatic effects of the binding of the SIBLINGs to proMMPs, a fluorescent substrate (casein- or gelatin-fluorescein conjugate) assay was employed to screen the protease activity of the proMMPs. ProMMP-2, proMMP-3, and proMMP-9 were titrated with increasing concentrations of all three SIBLINGs or with vehicle alone and the resulting enzyme activity measured by increased fluorescence signal. Increased proteolytic activity was observed only for the three strong proMMP-SIBLING binding pairs and a graded dose–response was evident (Fig. 6A, B, and C). When the strong binding SIBLING was added to its corresponding active MMP, enzymatic activity was not significantly changed (Fig. 6D, E, and F). This suggests that the binding of the SIBLING with its active MMP partner did not interfere with its normal proteolytic activity.

The properly matched SIBLING–proMMP pairs showed a dose–response increase in the amount of gelatin (proMMP-2 and proMMP-9) or casein (proMMP-3) digested (Fig. 6G, H, and I). Incubation of SIBLING with substrate was no different from substrate alone, showing that the increase in activity in the proMMP + SIBLING was not caused by any proteolytic activity in the SIBLING preparations used for these experiments. Mismatched pairs of SIBLINGs and proMMPs (BSP with proMMP-3 and proMMP-9, OPN with proMMP-2 and proMMP-9, and DMP1 with proMMP-2 and proMMP-3) analyzed under identical conditions each yielded an activity (as measured by relative change in fluorescence over time) equivalent to that of the residual activity of each commercial proMMPs alone (Fig. 6J, K, and L). Given that there was no observed increase in the amount of propeptide-free enzyme in all of these SIBLING-proMMP pairs (see above), it is reasonable to hypothesize that the increase in activity is due to a conformational change in the protease that allows its propeptide to be removed from the active site and thereby permit the digestion of both small and large macromolecular substrates.

SIBLINGs restore activity to inhibited MMPs

The quenching of tryptophan fluorescence and the increase in activity caused by SIBLING binding to the proMMP are consistent with an alteration in the local structure near the active site. The addition of BSP increased the activity of proMMP-2 more than twofold, whereas addition of

the specific inhibitor caused a reduction in the basal activity (Fig. 7A). The activity of the proMMP-2 + BSP complex treated with equimolar amounts of the inhibitor was 84% of that shown by the SIBLING + proMMP complex alone and sixfold higher than that of proMMP + inhibitor. That the increase in proMMP activity did not arise from activity copurifying with BSP was indicated by the lack of additional activity seen upon addition of BSP alone to the assay. Similarly, the abilities of the proMMP-3+OPN complex and the proMMP-9+DMP1 complex to be inhibited by equimolar amounts of their specific MMP inhibitors resulted in only a 20–25% reduction in the maximum SIBLING-stimulated rate (Fig. 7D and G). As was the case for BSP, the relative rate of change in fluorescence was not significantly different between proMMP, SIBLING, or inhibitor controls. In contrast to the specific inhibitors, 1,10 phenanthroline (which disrupts MMP activity by chelating and removing the active site required zinc ion) blocked proMMP activity even in the presence of SIBLING.

The active forms of the MMPs also exhibited quenching of tryptophan fluorescence emission upon binding their specific SIBLING partner. The possibility that SIBLING binding also altered inhibitor interaction with active MMPs was investigated. Specific low-molecular-weight inhibitors were again used to block active MMP activity on the fluorescein-labeled substrate in the presence of SIBLINGs. MMPs were incubated with either 1) vehicle, 2) equimolar SIBLING, 3) equimolar MMP-specific inhibitor, 4) MMP-specific inhibitor and SIBLING, or 5) SIBLING + 1,10 phenanthroline. The addition of SIBLING caused no significant change in MMP activity, whereas the inhibitor-treated MMP exhibited an expected dramatic loss of activity (Fig. 7B, E, and H). The addition of the corresponding SIBLING, however, rescued much of the original activity, even in the presence of equimolar amounts of specific inhibitor. As was the case for proMMPs, SIBLINGs were not able to restore activity to active MMPs treated with 1,10 phenanthroline.

The effect of SIBLING on the ability of the specific inhibitors to decrease MMP enzyme activity was further investigated by studying the dose–response. The activity of authentic active MMPs + equimolar SIBLINGs were measured using the fluorescein-gelatin or casein fluorescent assay in the presence of increasing concentrations of the MMP-specific inhibitors. SIBLINGs did not alter active MMP activity, whereas the addition of 1.6 nM (equimolar) MMP-specific inhibitor to MMP alone decreased activity dramatically. When the complex of equimolar active MMP + SIBLING was treated with increasing concentrations of the inhibitor, significant loss of activity was observed but only at substantially higher concentrations (Fig. 7C, F, and I).

Because MMPs occur in vivo associated with TIMPs, the effect of SIBLINGs on the activity of MMP + TIMP complexes was investigated. Treatment of active MMP-2, MMP-3, or MMP-9 with equimolar amounts of the appropriate TIMP reduced the enzymatic activity of the MMP between 85 and 98% (Fig. 8). The addition of the correctly matched SIBLING to the TIMP-inhibited MMP caused a restoration of much of the original proteolytic activity. The addition of BSP to TIMP2-inhibited MMP-2 caused a fivefold increase in enzyme activity over that of the TIMP-MMP-2 complex alone. Similarly, the presence of equimolar OPN lead to a sevenfold increase in activity of TIMP1-inhibited MMP-3, whereas DMP1 restored TIMP1-inhibited MMP-9 activity over tenfold. It is a reasonable hypothesis that the conformational change in the active MMP upon binding its SIBLING partner lowers the affinity of the TIMP (and low-molecular-weight inhibitors) for the active site of the MMP thereby enabling substrate access.

Reversal of SIBLING-induced activity by Factor H

BSP, OPN and DMP1 have previously been shown to bind to Factor H with high affinity, 10–100 fold higher than that just described for their partner MMPs (10, 11). This binding has been shown in another context to be sufficiently strong to completely mask serum BSP and OPN in standard ELISA assays (23). The question arises as to whether Factor H can compete with MMPs for SIBLING binding and thereby affect each SIBLING's interactions with its respective proMMP and active MMPs. The gelatin and casein fluorescein conjugate assays were used to investigate whether TIMP-inhibited MMPs, which had regained enzymatic activity by the addition of their corresponding SIBLING could then have their enzymatic activity altered by the subsequent addition of purified Factor H. Addition of equimolar Factor H to samples containing SIBLINGs, activated MMPs and TIMPs caused a 75% reduction in the BSP-induced recovered activity for MMP-2, a 40% reduction for MMP-3 and a 90% reduction for MMP-9 (Fig. 8A, B, and C). The higher affinity of Factor H for the SIBLING protein appears to promote the removal of the SIBLING from the SIBLING-MMP complex thereby permitting the MMP to reverse its conformation and allow the TIMP to again bind to the active site and reinhibit the enzyme.

The action of Factor H on the SIBLING-mediated activation of proMMPs was also investigated. Reaction mixtures consisting of 1) proMMP alone, 2) proMMP + SIBLING, or 3) proMMP + SIBLING + Factor H were incubated with the fluorescein-gelatin conjugate and the fluorescent signal recorded for up to 8 h. The addition of Factor H caused the rate of substrate digestion by the SIBLING-activated proMMP complex to decrease suggesting that the removal of the SIBLING from the proMMP resulted in the reactivation of the catalytic activity by the still-attached propeptide (Fig. 8D, E, and F). The Factor H-mediated decrease in activity was observed for all three SIBLING-proMMP pairs consistent with the higher affinity Factor H binding and removing the SIBLING from the SIBLING-proMMP complexes. The addition of Factor H alone to the proMMPs or active MMPs had no effect on the observed activity (data not shown). These results support the hypothesis that the propeptide is not removed in order to create the enzymatic activity in the proMMP-SIBLING pairs.

DISCUSSION

In this study, we have demonstrated that three members of the SIBLING family (BSP, DMP1, and OPN) can each bind to different latent and the corresponding active MMPs (MMP-2, MMP-9, and MMP-3, respectively) in a 1:1 stoichiometry with nM affinity *in vitro*. This has been demonstrated by 1) copurification of the pairs through nondenaturing chromatographic columns; 2) solution phase intrinsic fluorescence binding studies; and 3) showing that BSP and OPN affinity columns can be used to purify MMP-2 and MMP-3, respectively, from media containing several MMPs. In addition, we have shown that purified SIBLINGs added to their respective authentic proMMPs cause an increase in proteolytic activity without removal of the inhibitory propeptide. In all of these observations, SIBLINGs and proMMPs showed specificity in their partnering, with BSP binding to and activating proMMP-2, OPN activating proMMP-3 and DMP1 activating proMMP-9. Other pairings of the SIBLINGs and proMMPs did not lead to binding saturation or to significant increases in proteolytic activity when studied in equimolar stoichiometries. Binding of the same preferred pairs of proteins was also seen for the active forms of the MMPs, although no changes in proteolytic activity against natural substrates were observed.

We have observed 1) fluorescent quenching during SIBLING binding titration (indicating a change in the microenvironment of the MMP's tryptophans); 2) increased susceptibility of proMMP-SIBLING pairs to plasmin digestion; 3) increased enzymatic activity of the proMMPs upon binding their specific SIBLING partner; 4) reduced ability of specific low-molecular-weight inhibitors to block SIBLING + MMP complexes; and 5) restoration of activity to TIMP-inhibited MMPs by the corresponding SIBLING. These observations are consistent with binding of SIBLING to MMP being associated with structural changes in the MMP. Our hypothesis is that these conformational changes induced in the structure of the MMP by the binding of the SIBLING partner can at least partially disrupt interaction of inhibitors with enzyme's active site, presumably by lowering the affinity of the tested inhibitors for their MMPs. In vivo, this result is likely to mean that even in the presence of TIMPs, MMPs may be enzymatically active in regions of locally high concentrations of specific SIBLINGs.

Complement Factor H, a high-abundance serum protein known to have a high affinity for these three SIBLINGs, was shown to reverse the SIBLING-induced resistance of the active MMP to TIMPs, presumably by binding and removing the SIBLING partner from the complex. The decrease by Factor H of SIBLING-mediated proMMP activation is also consistent with competition between the MMP and Factor H for the binding of the SIBLING. That a portion of the activity of the three proMMP-SIBLING pairs returned to their normal low levels upon the addition of Factor H would suggest that the propeptide can reinsert into the active site and return the protease to its inactive state. However, that some enzymatic activity remains after the addition of the Factor H also suggests that the propeptide reinsertion is not always successful under these specific in vitro conditions. Factor H invariably prevents the active complexes from forming when it binds to the SIBLINGs before they can complex with their partnering MMPs. Our previous work had indicated that these three SIBLING family members in solution were rapidly bound by complement Factor H. This suggests that the activation of the proMMP or reactivation of TIMP-inhibited MMPs by the simple binding of their respective SIBLINGs will be limited to short distances from their sites of secretion due to the abundance of Factor H in the body.

In vivo, the proforms of the MMPs are generally fully activated through proteolytic cleavage of the inhibitory propeptide. Interestingly, TIMP-2, a protein that can inhibit the catalytic activity of MMP-2, is also thought to play a role in at least one mechanism of activation of propeptide cleavage (28). This activation of proMMP-2 has been proposed to involve formation of a complex of the proMMP-2 with TIMP-2 and MT1-MMP (MMP-14) on the cell surface. A second, TIMP-2-free, MT1-MMP then activates the MMP-2 by cleaving off the propeptide. By analogy, it is interesting to hypothesize that proMMPs undergoing a conformational change when binding their SIBLING partners may become better substrates for other proteases in vivo that result in the removal of the inhibitory propeptides. This paper has shown such an increase in susceptibility to at least one protease, plasmin.

Finally, because the expression of two of these SIBLINGs—BSP and OPN—have been correlated with cancer progression and severity, it is interesting to consider that these proteins may be locally activating their corresponding proteases in vivo. All three MMPs have been shown to be important in tumor development and metastasis (24). The modulation of MMP activity by SIBLINGs may even include SIBLINGs coming from the tumor cells and the MMPs from the nearby stroma. Furthermore, because MMPs have been shown to be associated with vascular invasion (29, 30) and that BSP and OPN have been shown to possess angiogenesis

activity in vivo (31, 32), it may be that the two observations can be linked by the mechanism of MMP activation presented in this report. By defining the basic biochemistry of these interactions, we now possess the requisite structural knowledge and analytical tools to pursue meaningful tissue culture and in vivo studies.

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Fig. 1

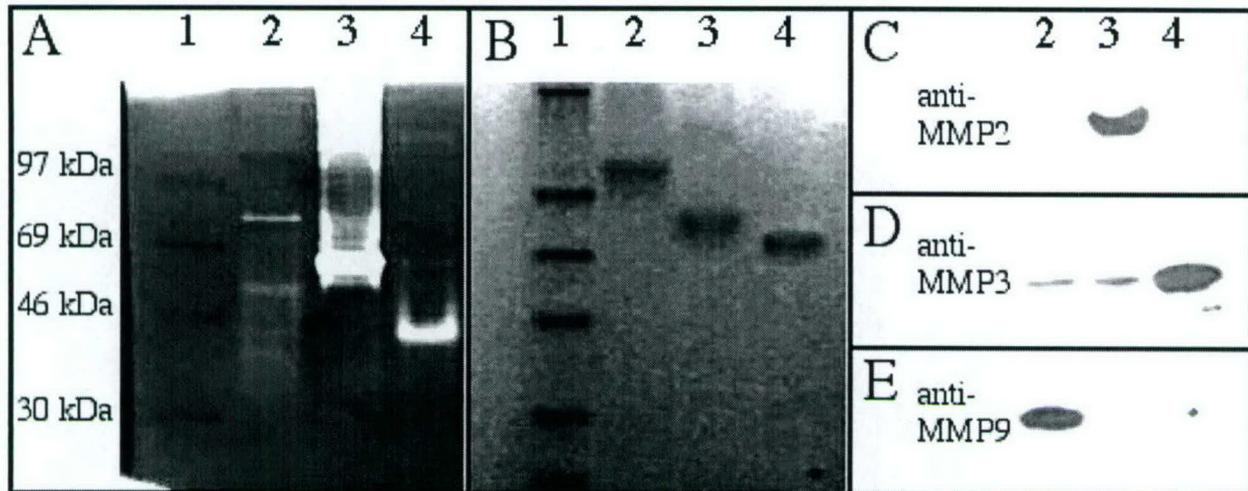


Figure 1. MMPs copurify with SIBLINGs. Recombinant proteins were purified from essentially identical culture media sources under nonreducing conditions as described (11). Ten micrograms of each SIBLING were run on a casein zymogram gel. After electrophoresis, the gel was processed for zymography. (A) The gel was stained with Coomassie Blue and visualized by dynamic integrated exposure. (B) The destained gel was restained with StainsAll to visualize the acidic SIBLINGs. Lane 1, molecular weight standards; lane 2, DMP1; lane 3, BSP; lane 4, OPN. A 12% acrylamide gel was loaded with the same samples, electrophoresed, transferred to nitrocellulose membranes, probed with anti-MMP-2 (C), anti-MMP-3 (D) or anti-MMP-9 (E) and detected by chemiluminescence.

Fig. 2

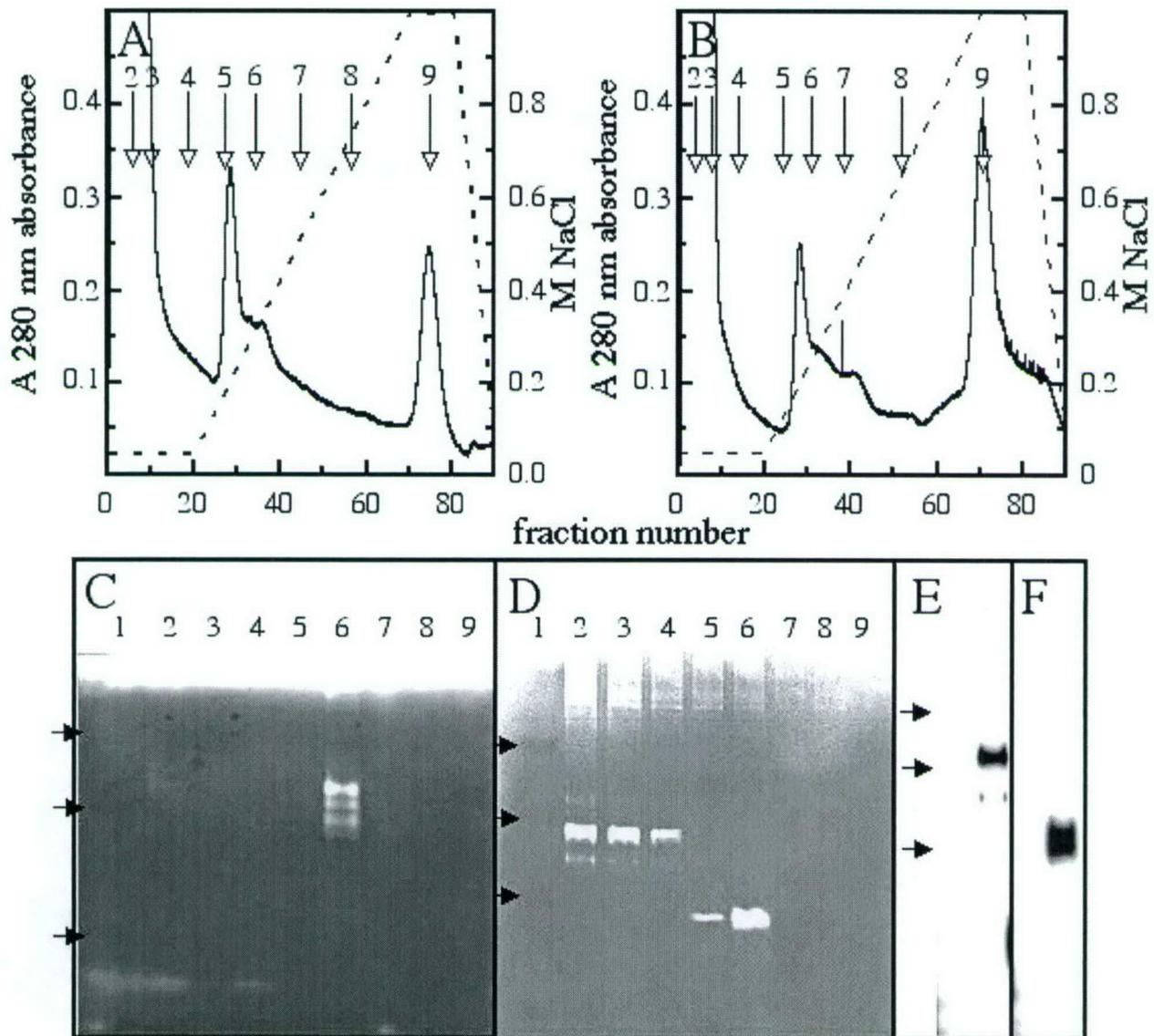


Figure 2. Affinity purification of specific MMPs on SIBLING columns. Serum-free conditioned medium from B16F10 cells was loaded onto the BSP-affinity column (A) or OPN-affinity column (B) and washed until the absorbance at 280 nm returned to background. A linear salt gradient was used to elute bound material. Fractions corresponding to numbered arrows were analyzed by gelatin (C) or casein (D) zymography. Lane 1, molecular weight standards; lanes 2 through 9 correspond to fraction numbers in panels A and B. The UV-absorbing peak at 1 M salt is an apparent buffer absorbance artifact, as no protein was evident on SDS PAGE (lane 9). 12% acrylamide gel was loaded with aliquots of fraction 6 from each affinity column, electrophoresed and transferred to nitrocellulose membranes. Fraction 6 from the BSP affinity column was probed with anti-MMP-2 (E), while fraction 6 from the OPN affinity column was probed with anti-MMP-3 (F), and the immunoreactive material detected by chemiluminescence. The migration positions of the 97, 69, and 46 kDa protein standards are marked by the arrows.

Fig. 3

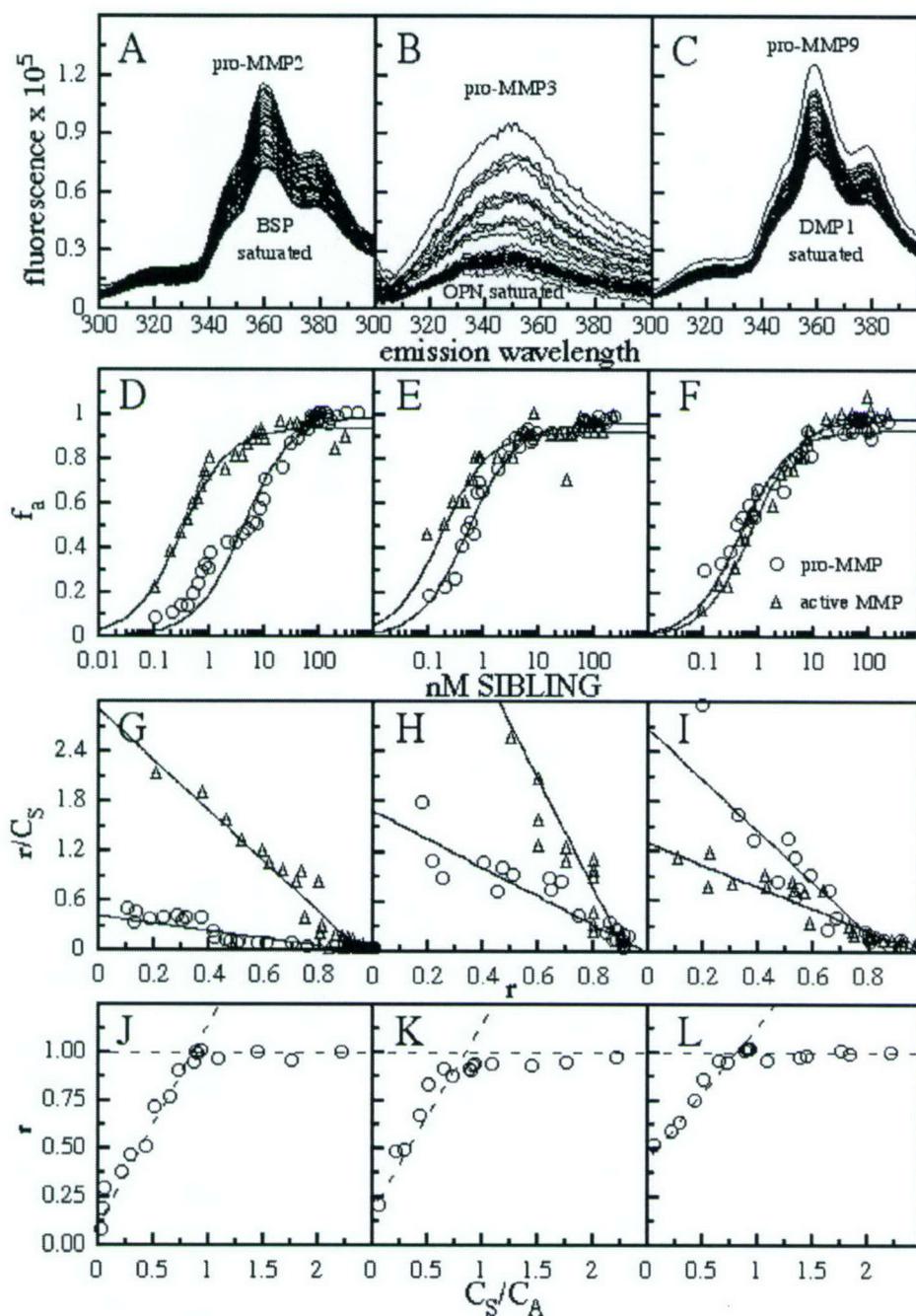


Figure 3. SIBLING binding to MMPs by intrinsic fluorescence titration. Binding interactions were investigated by titrating 3.5 nM (A) proMMP-2, (B) proMMP-3, and (C) proMMP-9 with BSP, OPN, or DMP1, respectively. The areas under the emission peak curves were used to determine binding functions by calculating fractional acceptor saturation of proMMP-2 (O) or active (Δ) MMP-2 with BSP (D), proMMP-3 (O) or active (Δ) MMP-3 with OPN (E), and proMMP-9 (O) or active (Δ) MMP-9 with DMP1 (F). Scatchard plots of proMMP-2 and active MMP-2 with BSP (G), proMMP-3 and active MMP-3 with OPN (H), as well as proMMP-9 and active MMP-9 with DMP1 (I) were determined. The stoichiometry was defined for BSP and proMMP-2 (J), OPN and proMMP-3 (K), and DMP1 and proMMP-9 (L) as described in Experimental Procedures.

Fig. 4

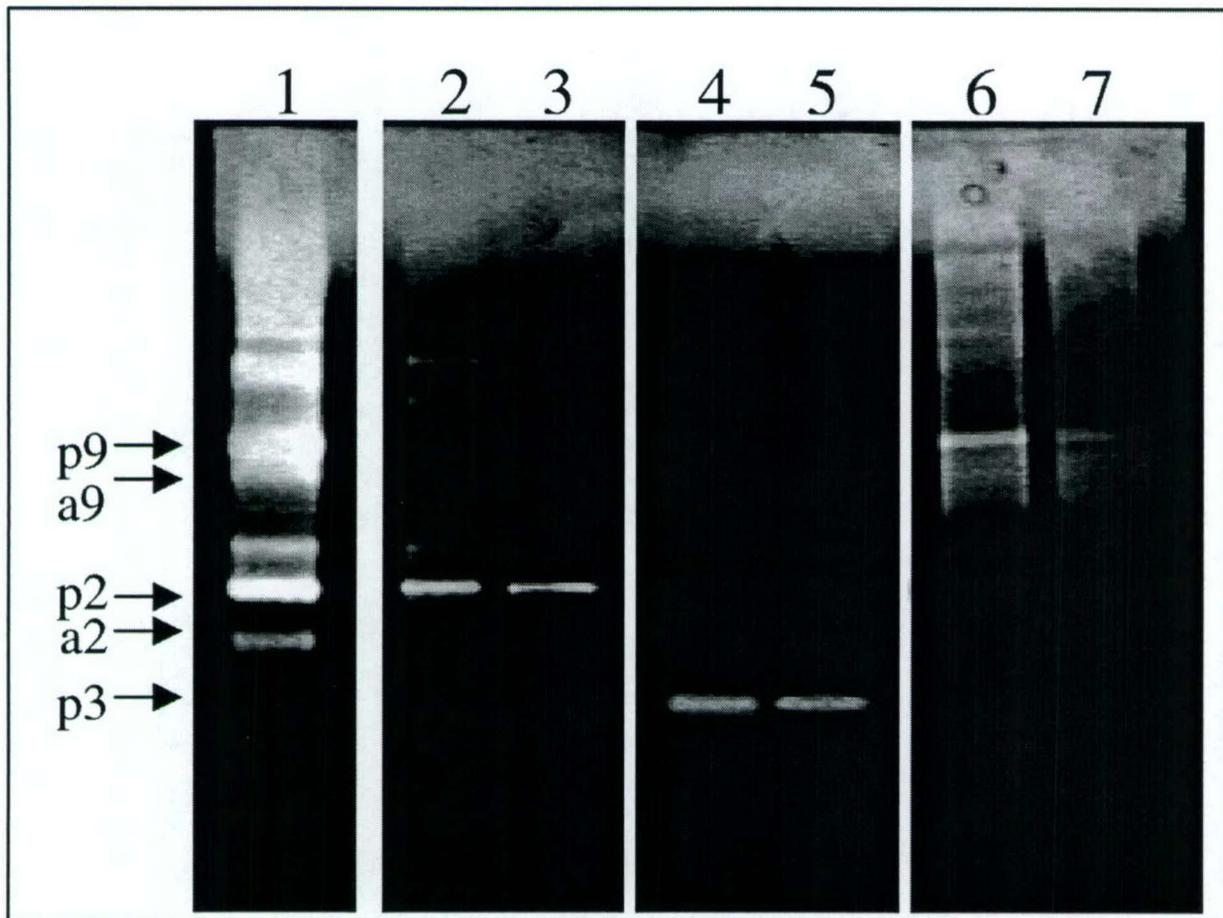


Figure 4. SIBLINGs bind but do not result in significant auto-cleavage of the MMP propeptides. Equivalent nanomolar quantities (140 nM) of correctly matched latent MMPs and SIBLINGs were incubated for 1 h and resolved by zymography to monitor propeptide presence and relative zymogen levels. ProMMP-2 and proMMP-9 samples were resolved by gelatin zymography (lanes 1, 2, 3, 6, and 7) while proMMP-3 containing samples were resolved by casein zymography (lanes 4 and 5). Lane 1, MMP standards; lane 2, proMMP-2; lane 3, proMMP-2 + BSP; lane 4, proMMP-3; lane 5, proMMP-3 + OPN; lane 6, proMMP-9; lane 7, proMMP-9 + DMP1. Abbreviations, p2, proMMP-2; a2, active MMP2; p3, proMMP-3; a3, active MMP-3; p9, proMMP-9; a9; active MMP9.

Fig. 5

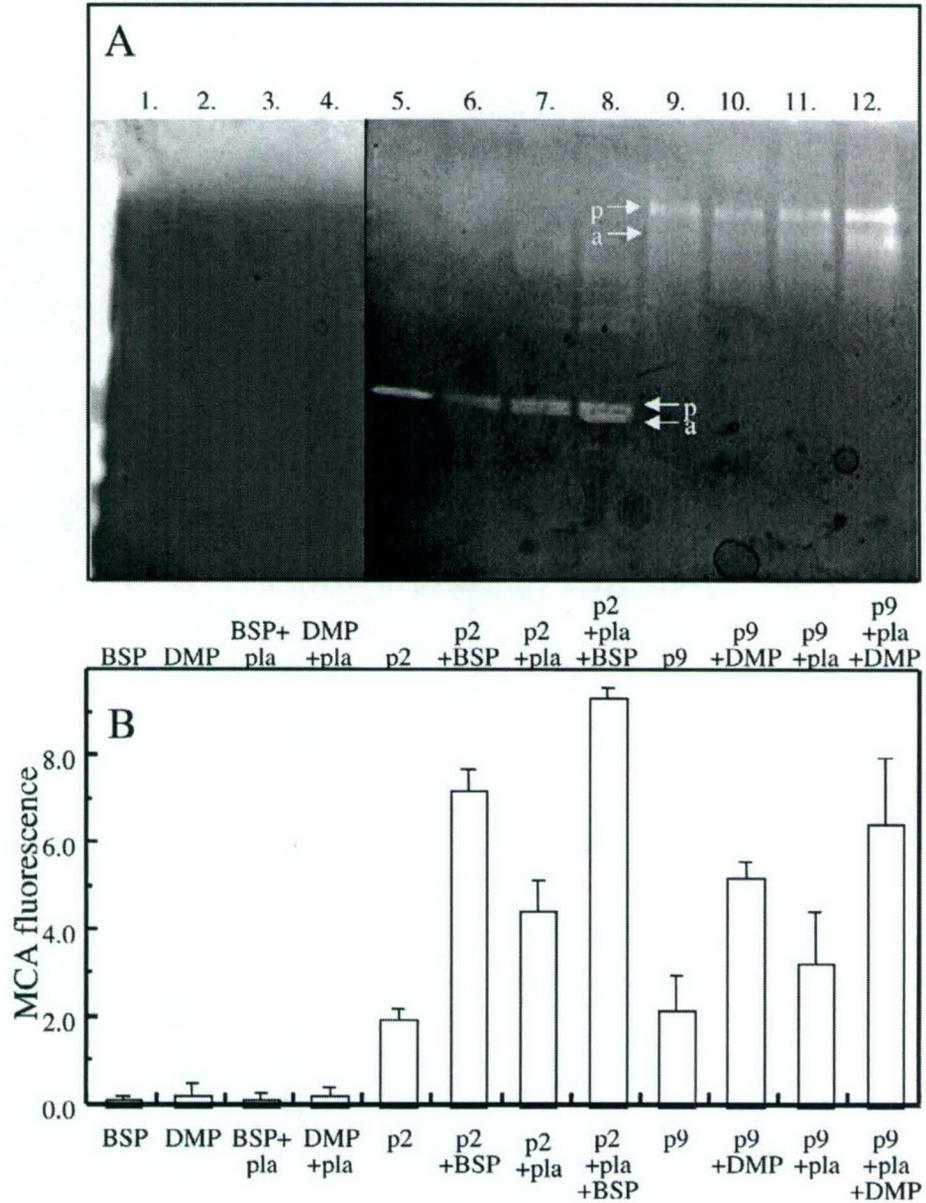


Figure 5. SIBLING–MMP complexes have altered plasmin susceptibility. Changes to the structure of MMPs caused by SIBLING binding were studied by determining the enzyme susceptibility of the MMP–SIBLING complex to digestion with plasmin. As negative controls, BSP (lane 1), DMP1 (lane 2), BSP + plasmin (lane 3) and DMP1 + plasmin were incubated for 15 min at 37 C and analyzed by zymography. In addition, latent 140 nM MMP-2 was incubated alone (lane 5), with equimolar BSP (lane 6), with plasmin (lane 7) or with equimolar BSP followed by plasmin (lane 8), while 140 nM proMMP-9 was incubated either alone (lane 9), with equimolar DMP1 (lane 10), with plasmin (lane 11) or with equimolar DMP1 followed by plasmin (lane 12) for 15 min. The samples were resolved by zymography (A). Note the appearance of significant amounts of a slightly lower band (“active” MMP) only in the plasmin-treated samples containing the SIBLING partners. Abbreviations, p2, proMMP-2; p9; proMMP-9; pla, plasmin. Arrowheads indicate migration position of proMMP (p) and MMP (a) The enzymatic activities of the same 12 conditions were also determined after 15 min incubation by measuring the relative fluorescent yield from small molecular weight MMP-specific substrates as described in Experimental Procedures (B).

Fig. 6

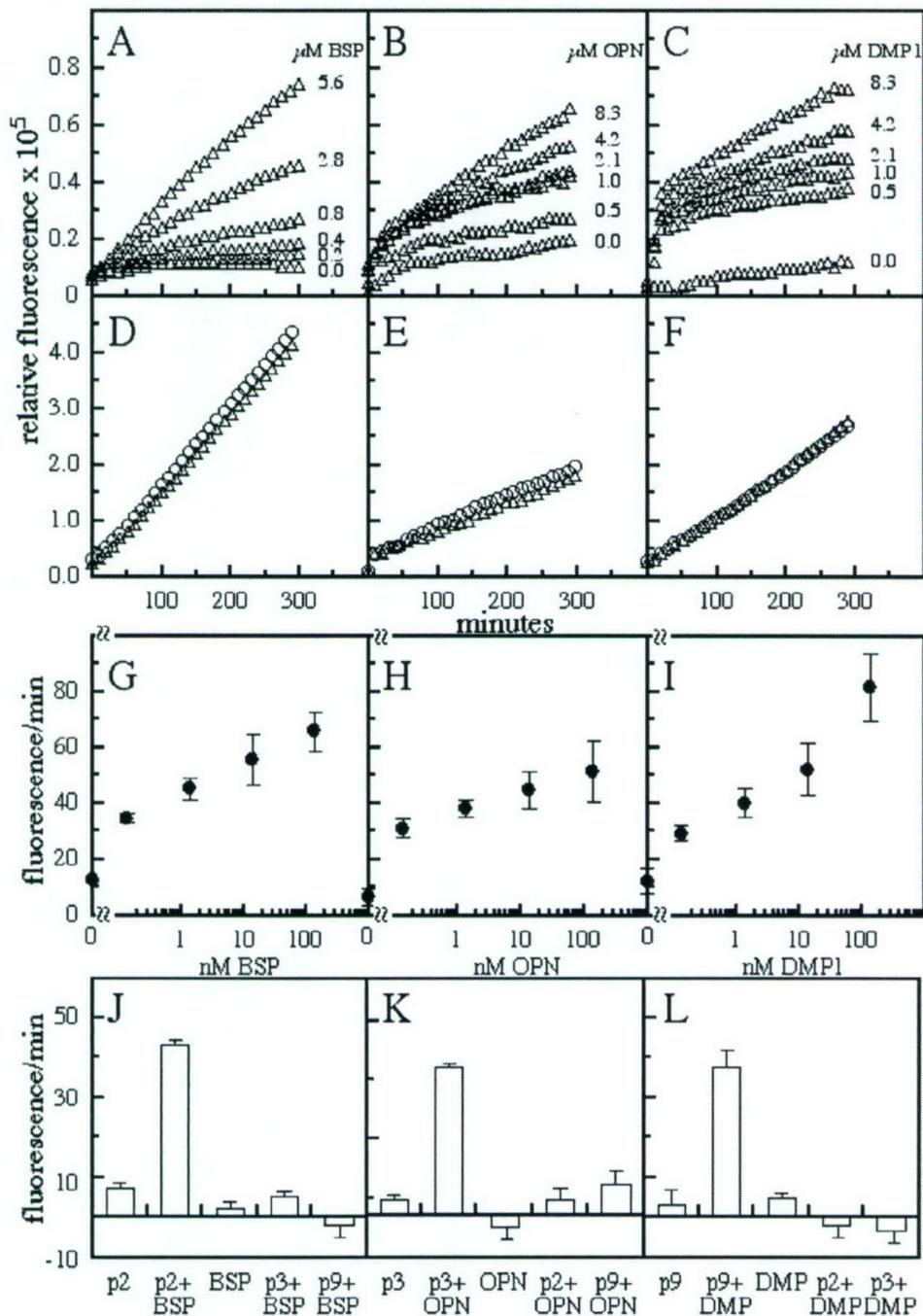


Figure 6. Modulation of MMP activity by SIBLINGS. Protease activity was followed by incubating fluorescent substrate with 1.4 nM proMMP-2 (A), proMMP-3 (B), and proMMP-9 (C) and increasing concentrations of BSP, OPN, or DMP1, respectively. The activity of equimolar concentrations (1.4 nM) of active MMP-2 \pm BSP (D), MMP-3 \pm OPN (E), and MMP-9 \pm DMP1 (F) in the same assay showed no difference. The same assay was also employed to determine a low SIBLING concentration dose-response in activity of proMMP-2 + BSP (G), proMMP-3 + OPN (H), and proMMP-9 + DMP1 (I). The activity of 1.4 nM BSP (J), OPN (K) and DMP1 (L) with correctly matched proMMPs and mismatched proMMPs as well as of proMMPs or SIBLINGS alone were analyzed by determining the change in fluorescence/minute over the first 3 h of incubation. Values plotted represent the mean of three combined experiments \pm standard deviation.

Fig. 7

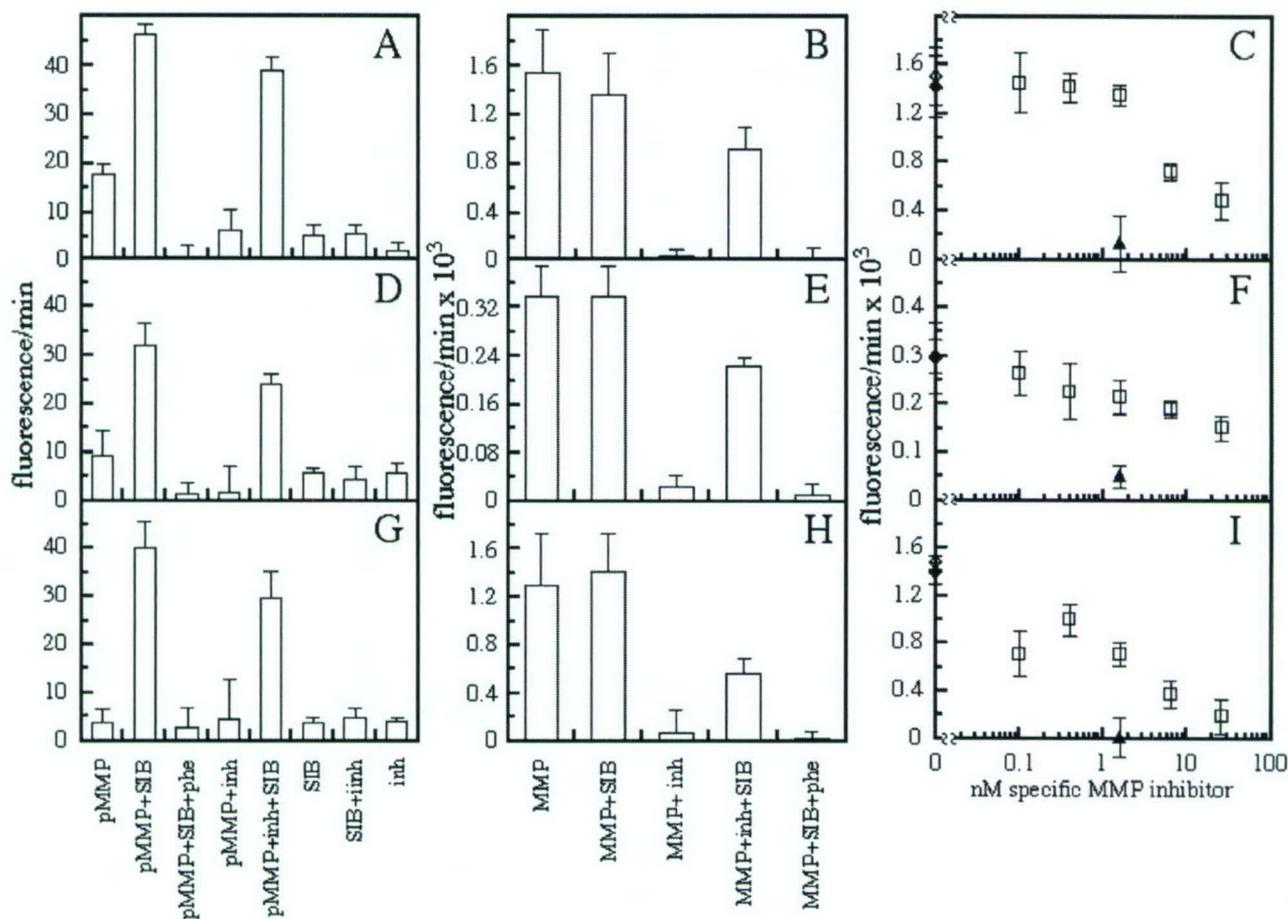


Figure 7. The induced enzymatic activity of MMPs bound with matching SIBLING partners displays altered inhibition by specific low molecular weight inhibitors of MMPs. The ability of the inhibitors to block the SIBLING-activated MMPs was tested using the fluorescein-labeled substrate assay. Substrate was incubated with 1.4 nM MMP alone, MMP + equimolar SIBLING, MMP + equimolar MMP-specific inhibitor, MMP + equimolar MMP-specific inhibitor + equimolar SIBLING, or 1.4 nM MMP + equimolar SIBLING + 1,10 phenanthroline. The relative rates of substrate cleavage compared by plotting the average fluorescent change/min are summarized for BSP with proMMP-2 (**A**) or active MMP-2 (**B**), OPN with proMMP-3 (**D**) or active MMP-3 (**E**), and DMP1 with proMMP-9 (**G**) or active MMP-9 (**H**). Values plotted as bars are the mean of four replicates and standard deviations. Abbreviations: pMMP, proMMP; SIB, SIBLING; phe, 1,10 phenanthroline; inh, MMP-specific inhibitor (as described in Materials and Methods). The effect of SIBLING binding was further studied by titration of active MMP + SIBLING complexes with increasing doses of MMP-specific inhibitor for BSP and MMP-2 (**C**), OPN and MMP-3 (**F**), DMP1 and MMP-9 (**I**). For (**C**), (**F**) and (**I**), the symbols represent: open diamond, activity of MMP alone; solid diamond, activity of MMP + SIBLING; solid triangle, activity of MMP + equimolar specific inhibitor; and open square, activity of MMP + SIBLING + specific inhibitor.

Fig. 8

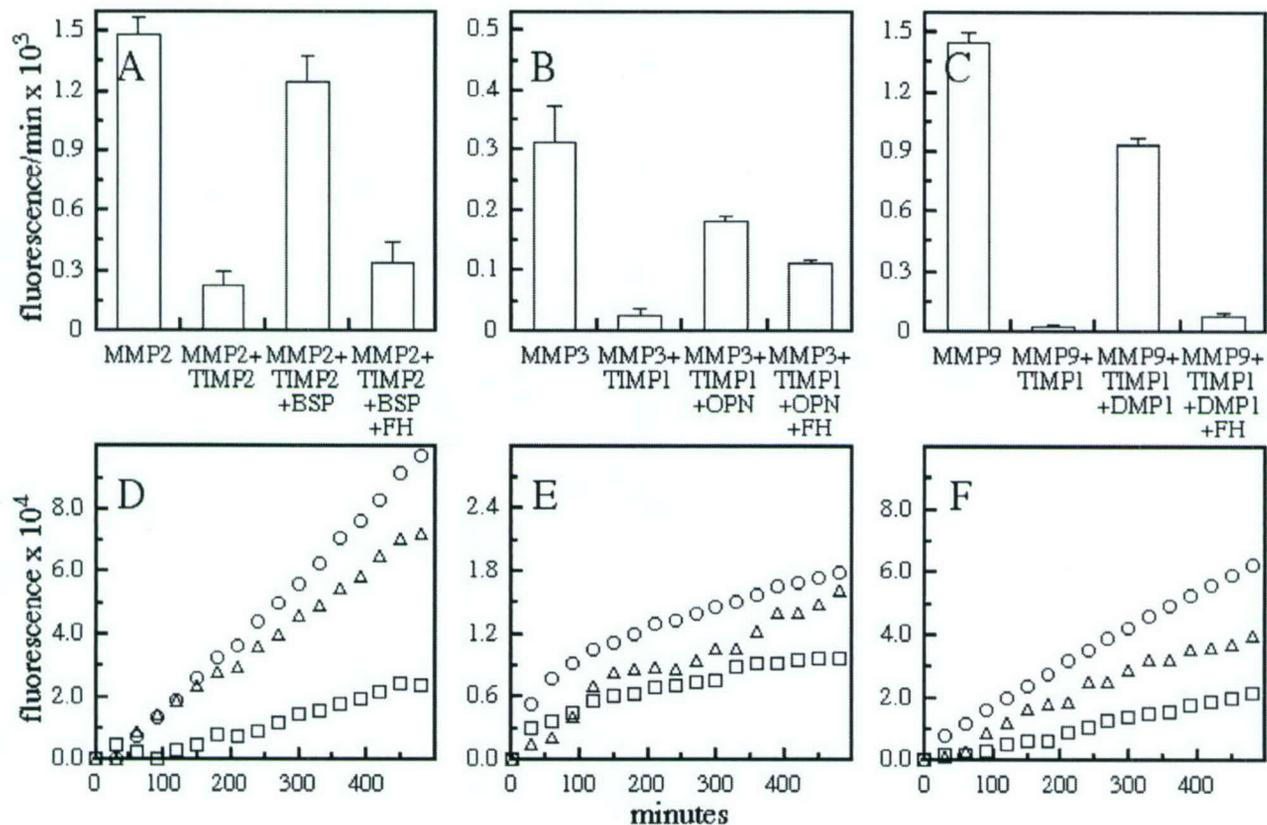


Figure 8. TIMP and Factor H modulation of SIBLING-mediated MMP activation. Active MMP-2 complexed with equimolar TIMP2 and active MMP-3 or MMP-9 complexed with equimolar TIMP1 were incubated for up to 15 h with vehicle, equimolar SIBLINGs, or equimolar SIBLINGs followed by equimolar Factor H (1.4 nM each) in the presence of the fluorescein-gelatin conjugate (**A** and **C**) or fluorescein-casein (**B**). The data was analyzed by linear regression analysis over the first 3 h and the slope (fluorescent change/minute) determined. The relative rates of MMP activity were compared by plotting the average of duplicate analyses of fluorescent change/min \pm the standard error of the slope for each reaction mixture composition. The ability of Factor H to reverse the SIBLING-mediated activation of proMMPs was investigated using the same assay. 1.4 nM proMMP was incubated with vehicle, equimolar SIBLING, or equimolar SIBLING + Factor H. The equimolar mixtures were BSP and proMMP-2 (**D**), OPN and proMMP-3 (**E**), and DMP1 and proMMP-9 (**F**), where square = proMMP + vehicle; circle = proMMP + SIBLING; and triangle = proMMP + SIBLING + Factor H. Abbreviations: FH, Factor H.

Appendix II

Serum Levels of Matrix Extracellular Phosphoglycoprotein (MEPE) in Normal Humans Correlate with Serum Phosphorus, Parathyroid Hormone and Bone Mineral Density.

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ABSTRACT. Matrix extracellular phosphoglycoprotein (MEPE), a member of the Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family, is primarily expressed in normal bone and has been proposed as a phosphaturic factor because of high expression and secretion in oncogenic hypophosphatemic osteomalacia tumors. In order to begin to address the role of MEPE in normal human physiology, we developed a competitive ELISA to measure serum levels of MEPE. The ELISA was used to characterize the distribution pattern in a population

consisting of 114 normal adult subjects. The mean value of MEPE was 476 ± 247 ng/ml and levels decreased significantly with increasing age. MEPE levels were also significantly correlated with serum phosphorus and parathyroid hormone (PTH). In addition, MEPE levels correlated significantly with measures of bone mineral density in the femoral neck and total hip in a subset of 60 elderly subjects. The results are consistent with MEPE being involved in phosphate and bone metabolism in a normal population.

Introduction

Matrix extracellular phosphoglycoprotein (MEPE), is a member of the SIBLING gene family (1, 2). Other family members include bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein-1 (DMP1), and dentin sialophosphoprotein. The family shares the RGD integrin-binding motif, several conserved phosphorylation and N-glycosylation sites, a common gene structure and chromosomal localization (4q21).

Normal MEPE expression has been described primarily in bone marrow, brain (3) and bone (4), while tumors which cause hypophosphatemic osteomalacia exhibit high expression and secretion (3). MEPE has consistently been linked with mineralization and bone formation associated with bone mineral (3-6). Whether MEPE plays a role as a positive or negative regulator of bone formation in humans remains unclear. The current study was undertaken to determine the distribution of MEPE in normal donors and to correlate the values with other biomarkers of bone metabolism as well as measures of bone mineral density (BMD).

Methods

Subjects. Sera from clinically defined normal patients were obtained under IRB approved protocols from a commercial serum bank (East Coast Biologicals, Inc., North Burwick, ME) as well as from the Johns Hopkins Bayview Medical Center General Clinical Research Center (JHBMC). The JHBMC normal group was obtained from an existing serum bank using samples from which all patient identifiers were removed.

Cloning and expression of MEPE. The last exon of human MEPE constitutes 95% of the mature protein as defined by Rowe et al. (3). The last exon was amplified by PCR from human genomic DNA using a 5' oligonucleotide with a NdeI restriction site engineered in (AGTACCCATATGAAAGACAATA-TTGGTTTCACCAT) and a 3' oligonucleotide with a BamHI site (CTGATGGGATCCCTAGTCACCAT-CGCTCTCAC). The ~1.5 kbp PCR product was gel purified, digested with NdeI plus BamHI, ligated into pET15b expression vector (Novagen, Madison, WI) digested with the same restriction enzymes. After transfection into BL-21 (DE3) E. coli cells, a high expression colony was selected and used to produce the MEPE protein by stimulation with IPTG. The MEPE protein in the apparent inclusion bodies was purified in 6 M urea on a HisBind resin column (Novagen) following the manufacturer's instructions. The eluted fraction was dialyzed exhaustively against 0.1 M ammonium acetate at 4°C and freeze-dried. Four ~200 µg aliquots of the highly purified MEPE were injected into a New Zealand white rabbit to make antiserum LF-155.

Serum sample preparation and competitive ELISA procedure. The SIBLINGs BSP, OPN and DMP1 are complexed with complement Factor H in human serum (7, 8). We have developed competitive ELISAs for measuring the BSP and OPN that requires disruption of the serum complex between the SIBLING and complement Factor H (9). For the current study, serum samples for use in ELISA analyses were processed in a chaotropic buffer exactly as described (9). The MEPE competitive ELISA developed utilized the same plates, buffers, and protein standard concentrations, secondary antibody concentrations, as well as substrate color reagents as

previously described (9). The only changes to the ELISA steps were that plates were coated with 10 ng/well recombinant MEPE and the primary antibody, LF-155, was employed at a 1:200,000 dilution.

Western blotting. Samples diluted in gel sample buffer were resolved by Tris/glycine SDS 12% polyacrylamide gels (Invitrogen, Inc., Carlsbad, CA) and transferred to nitrocellulose following standard conditions (10). Nitrocellulose membranes were rinsed with Tris-buffered saline-Tween (TBS-Tween, 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl containing 0.05% Tween 20). After a 1 h incubation in TBS-Tween + 5% non-fat powdered milk at room temperature, a 1:20,000 primary antibody (polyclonal antibody LF-155) was incubated overnight at 4 °C. The blot was washed in TBS-Tween four times for 5 min with TBS-Tween and then HRP-conjugated goat anti-rabbit IgG (1:2,000) in TBS-Tween + 5% milk was added and incubated for 2 h at room temperature. After washing, enhanced chemiluminescence reagents were employed for signal detection (Pierce Chemical Co., Chicago, IL) with x-ray film.

DEXA measurements. BMD was measured in the hip, spine and proximal femur using the Hologic QDR 1000 scanner (Hologic Corp., Waltham MA). The precision of this machine is $1.8\% \pm 0.05\%$. Mean values for total hip and spine were obtained, as were BMD values for neck and trochanter in the left proximal femur.

Serum and urine biochemical measures. Blood samples were drawn in the morning after an overnight fast. Serum bone biochemical measurements included bone-specific alkaline phosphatase (Hybritech, San Diego, CA), osteocalcin (Immunotopics, San Clemente, CA), procollagen type I carboxy-terminal propeptide (DiaSorin Stillwater, MN), intact PTH (Nichols Institute, San Juan Capistrano, CA), and 25 hydroxy vitamin D (DiaSorin). The excretion of deoxypyridinoline crosslinks (Quidel Corp. San Diego, CA) and cross-linked amino-terminal telopeptides (OSTEX International, Seattle, WA) were assayed in 2-hour, second-void morning urine specimens. The values for cross-links were normalized to urinary creatinine assayed using the Jaffe Rate method and a Beckman Creatinine Analyzer 2 (11). Serum inorganic phosphorus was measured using standard clinical methods (12). The performance characteristics of the immunoassays as carried out in our laboratory are given in Table I.

Results

Previous work has demonstrated that the SIBLINGs BSP, DMP1 and OPN were bound to complement Factor H in serum (7, 8). Disruption of the serum complex required heating in a chaotropic buffer containing reducing agent, followed by a column step to clean up the sample (9). The same disruption procedure was used on serum samples from elderly and young adult donors and MEPE was detected by western blot. Immunoreactive bands

shifted in migration with reduction and younger donors appeared to have more MEPE present (Fig. 1a and b). The amount of MEPE present in sera from 114 different normal subjects was analyzed. A reproducible standard curve profile combining 34 different analyses performed over the past two years was obtained (Fig. 1c). MEPE values quantified by ELISA paralleled semi-quantitative results obtained from western blots (Fig/ 1c, inset).

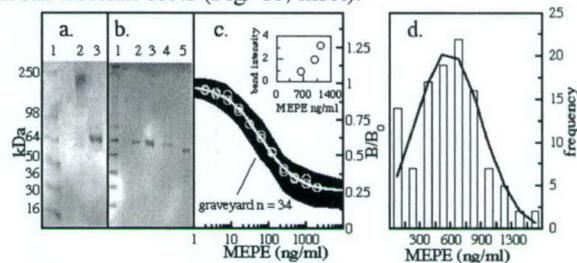


Figure 1. MEPE is present in human serum. (a) Unreduced (lane 2) and reduced (lane 3) serum samples from normal donors were analyzed for the presence of MEPE by western blot. Molecular weight standards were run in lane 1. (b) Samples derived from different age donors analyzed by western blot. Lane 1, standards; lanes 2, 3 & 4 contained 60, 24 and 78-year-old normal donor serum, lane 5, recombinant MEPE (lacking glycosylation). (c) Competitive ELISA profile; inset, representative correlation of western blot band intensity with ELISA results. (d) The distribution of MEPE (bars) in 114 normal subjects. Solid line represents the normal Gaussian distribution.

Addition of recombinant MEPE to serum samples prior to reduction, column chromatography and competitive ELISA yielded an average recovery of 88 % based on three different trials. The inter-assay coefficient of variance for repetitive measurements on the same serum sample was 19.4% (n = 6), while the intra-assay coefficient of variance was 12.6% (n = 12). The major source of this variance was tracked to the column chromatography step. Repeated measures of post-reduction and column samples gave rise to a coefficient of variance of 7.9%. The measure of MEPE levels in normal subject-derived sera revealed a distribution with a slight hook at the low end (Fig. 1d).

When MEPE levels were plotted versus the age of the subject, the reason for the low end hook to the distribution of normal values became apparent. MEPE exhibited a significant age-related decrease in level, (Fig. 2a). The population analyzed possessed a sufficient number of subjects > 60 years of age, where MEPE levels are 1/2 to 1/3 those of younger adults, to account for the increased distribution at low MEPE levels. Serum measures of markers of bone metabolism were also performed on normal subjects. Comparing MEPE levels with serum values of bone-specific alkaline phosphatase, procollagen type I carboxy-terminal propeptide, 25-hydroxy vitamin D, osteocalcin, and urine levels of collagen cross-link markers revealed no significant correlation (data not shown).

Table I. Immunoassay Performance and Study Population Characteristics.

Analyte	mean \pm s.d.	units	range	%CV intra-assay	%CV inter-assay
bone-specific alkaline phosphatase	11.2 \pm 4.2	ng/ml	5.0 - 28	5.49	5.83
deoxyypyridinoline crosslinks	5.1 \pm 2.0	nM/mM Cr	1.7 - 13	6.00	4.16
N-terminal telopeptides	31.3 \pm 13.3	BCE/mM Cr	5.2 - 64.2	8.25	4.00
osteocalcin	5.2 \pm 1.9	ng/ml	2.1 - 10.2	4.55	6.10
procollagen type I C-terminal propeptide	133.9 \pm 45	ng/ml	10.7 - 289	2.24	4.38
intact parathyroid hormone	33.7 \pm 14.5	pg/ml	3.2 - 94.2	2.40	5.95
25-hydroxy vitamin D	34.5 \pm 9.3	ng/ml	14.6 - 62.6	5.19	7.90
MEPE	476.0 \pm 247	ng/ml	19.0 - 1269	12.60	19.40
study					
male (n = 64)	60 \pm 20	years	21 - 87		
population					
female (n = 54)	55 \pm 12	years	35 - 62		
BMD group (n = 60)	65 \pm 11	years	50 - 82		

Because MEPE has been proposed to play a role in phosphate metabolism, we next investigated serum levels of PTH and inorganic phosphorus. Using a third generation commercial intact PTH assay, the PTH levels were found to be significantly negatively correlated with serum MEPE values (Fig. 2b). The serum levels of intact PTH showed no correlation with donor age (data not shown), suggesting that the association of MEPE and PTH was age-independent. The levels of serum phosphate in the same donors was significantly positively correlated ($r^2 = 0.35$, $p \leq 0.0001$) with serum MEPE values (Figure 2b, inset).

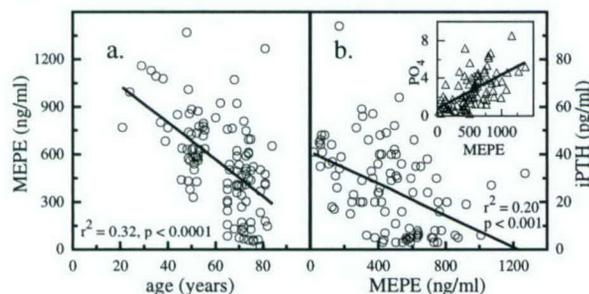


Figure 2. MEPE levels in serum correlate with age (a) and parathyroid hormone levels (b). Serum inorganic phosphorus levels were also determined (inset).

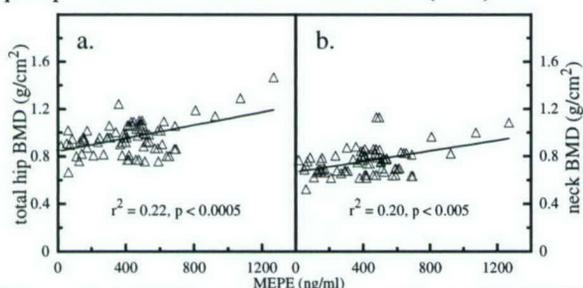


Figure 3. MEPE serum levels correlate with BMD values for total hip (a) and femur neck (b).

In addition to serum and urine markers of bone metabolism, BMD measurements were obtained on a subset of normal subjects ($n = 60$). The BMD values (g/cm^2) determined were analyzed for correlation with MEPE levels in the corresponding subject's serum (Fig. 3). MEPE levels were significantly positively correlated with bone mineral density values for total hip and femur neck. MEPE levels were also correlated with femur trochanter BMD ($r^2 = 0.13$, $p \leq 0.01$), while the correlation with total spine BMD did not reach statistical significance (data not shown). The correlation of serum MEPE

levels with BMD was still significant when adjusted for subject age using multiple regression analysis and StatView software (SAS Institute, Inc.)

Discussion

MEPE and its rodent homologue, OF45, have been implicated in bone and mineral metabolism (3, 4, 13). The increase in bone density found in the OF45 knockout mouse in the presence of normal serum phosphorus and calcium without evidence of a mineralization defect, suggests that it may have a direct effect on bone formation (6). In that report, the MEPE knockout animal had, what is for a gene knockout model, a relatively subtle increase in the amount of bone. While histomorphometric analysis was performed, there were no data on the parameters of mineralization (osteoid thickness, etc.), only formation and resorption. Elevated levels of MEPE mRNA expression by tumors from patients with hypophosphatemia and osteomalacia suggested that it may be involved in mineral homeostasis. The control of systemic phosphate homeostasis is incompletely understood. Key modulators include PTH, calcium, phosphorus, vitamin D, as well as novel phosphatonin(s), and the bone and kidney organs. Candidate phosphaturic factors include MEPE; PHEX, a putative endopeptidase believed to process factors regulating bone mineralization and renal phosphate reabsorption (5); FGF23, a phosphaturic factor in fibrous dysplasia (14), tumor-induced osteomalacia and autosomal-dominant hypophosphatemic rickets (15, 16) and secreted frizzled-related protein 4, an antagonist of renal Wnt-signaling (17). These phosphate regulators remain to be fully characterized both individually, and in their interactions which will lead to the description of a new hormonal pathway.

Demonstration of significant levels of MEPE in the serum of normal humans, as well as a clear age-related decrease suggest that MEPE may be an interesting marker of normal human bone and mineral metabolism. While the positive correlation between MEPE and phosphorus might suggest an anti-phosphaturic effect, it may represent a secondary response to higher serum phosphorus levels. This idea is supported by the significant correlation of serum MEPE levels with the important constituents of mineral metabolism serum, PTH and phosphorus. The relationships between serum MEPE and PTH, MEPE and phosphorus, and phosphorus and PTH are all internally consistent, and the relationship between phosphorus and PTH is consistent with established

physiology. The correlation of MEPE levels with BMD suggests that it may be involved in mineralization in the human. The finding that MEPE is low in aged patients, when BMD is lower, and that MEPE levels are higher when BMD is high is corroborative, and suggests that these findings in humans are of physiologic significance. Two recent studies have provided contrasting data on the biological activity of MEPE. Recombinant MEPE promoted renal phosphate excretion in mice and inhibited BMP2-mediated mineralization in a mouse osteoblasts cell line (18). The inhibitory action was mapped to the carboxy terminal region of the molecule. In a second study, a peptide fragment corresponding to the RGD-containing mid region stimulated new bone formation in neonatal mouse calvarial organ culture and increased osteoblast proliferation and alkaline phosphatase activity (19). Our current study demonstrates the association of serum MEPE levels with serum phosphate, PTH and bone mineral density but does not address causality.

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Appendix III

Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) gene family expression in different cancers.

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Running title: SIBLING expression and tumor progression.

Key words: bone sialoprotein, osteopontin, dentin matrix protein-1, biomarkers, progression.

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ABSTRACT

Purpose. Members of the SIBLING (Small Integrin Binding Ligand N-linked Glycoprotein) gene family have the capacity to bind and modulate the activity of matrix metalloproteinases (MMPs). The expression levels of five SIBLING gene family members – bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein (DMP1), matrix extracellular phosphoglycoprotein (MEPE), and dentin sialophosphoprotein (DSPP) as well as certain MMPs were determined using a commercial cancer array.

Experimental Design. Cancer profiling arrays containing normalized cDNA from both tumor and corresponding normal tissues from 241 individual patients were employed to screen for SIBLING and MMP expression in 9 distinct cancer types.

Results. Significantly elevated expression levels were observed for BSP in cancer of the breast, colon, stomach, rectum, thyroid and kidney; OPN in cancer of the breast, uterus, colon, ovary, lung, rectum, and thyroid; DMP1 in cancer of the breast, uterus, colon and lung; DSPP in breast and lung cancer. The degree of correlation between a SIBLING and its partner MMP was found to be significant within a given cancer type (e.g. BSP and MMP-2 in colon cancer, OPN and MMP-3 in ovarian cancer; DMP1 and MMP-9 in lung cancer). The expression levels of SIBLINGs were distinct within subtypes of cancer (e.g. breast ductal tumors compared to lobular tumors). In general, SIBLING expression increased with cancer stage for breast, colon, lung and rectal cancer.

Conclusions. These results suggest SIBLINGs as potential markers of early disease progression in a number of different cancer types, some of which currently lack vigorous clinical markers.

INTRODUCTION

The SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) gene family is clustered on human chromosome 4 and its members include bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein (DMP1), matrix extracellular phosphoglycoprotein (MEPE), and dentin sialophosphoprotein (DSPP) (1). SIBLINGs are normally thought to be restricted in expression to mineralizing tissue such as bones and teeth (1). OPN and BSP are also produced by trophoblasts (2, 3) and induced in certain neoplasms (4-12). Retrospective studies using pathological specimens have shown that BSP and OPN are expressed by breast (5, 11, 13-16), prostate (17, 18), lung (12), and thyroid (10, 19) cancers. DMP1 has been shown to be strongly up-regulated in lung cancer (20). Elevated levels of MEPE mRNA expression by tumors from patients with hypophosphatemia and osteomalacia have been reported (21). The neoplastic expression pattern of DSPP has not been defined.

Matrix metalloproteinases (MMPs) are critical for development, wound healing, and for the progression of cancer. We have recently shown that BSP, OPN and DMP1 specifically bind to proMMP-2, proMMP-3 and proMMP-9, respectively, thereby activating the latent proteolytic activity (22). Furthermore, it was shown that active MMPs inhibited by either tissue inhibitors of MMPs (TIMPs) or low molecular weight synthetic inhibitors were reactivated by their corresponding SIBLING. The current study was undertaken to determine the mRNA expression patterns of SIBLINGs in 9 different types of cancer. An additional goal was to determine whether SIBLINGs exhibited expression levels that correlated with their MMP partners as well as various measures of tumor progression.

MATERIALS AND METHODS

Cancer array analysis.

A cancer profiling array (product # 7841-1, Clontech, Palo Alto, CA) containing normalized cDNA from tumor and corresponding normal tissues from 241 individual patients was employed to screen for SIBLING and MMP expression (23). Several cancer profiling arrays were hybridized in ExpressHyb hybridization solution (Clontech) with ³²P-labeled cDNA probes as per the manufacture's instructions. Briefly, 1-2x10⁷ cpm of random-prime labeled cDNA was made single stranded by heating to 95 C for 5 min. and allowed to hybridize with the prepared membrane overnight at 65 C. Membranes were washed in a series of high stringency washes as recommended by the manufacturer. The washed membranes were quantified by exposure to PhosphorImager screens for one to 24 hours and the exposed screen analyzed on a Molecular Dynamics PhosphorImager (Amersham Biosciences Corp, Piscataway, NJ) using the manufacturer's ImageQuant program. All PCR products were subcloned into a shuttle plasmid, cloned, sequenced, and the inserts were gel-purified prior to labeling with ³²P-labeling by random priming. Unincorporated label was removed prior to hybridization.

SIBLING probes.

The labeled DNA used for probing was obtained as follows. Human BSP and OPN were cDNA inserts released from OP-10 and B6-6g plasmids respectively (24, 25). Human DMP1 insert was the ~1.4 kbp coding region of exon 6 (26) amplified from human genomic DNA subcloned into pBluescript at the EcoRI and BamHI sites using

oligonucleotides, ATTATAGAATTCAAATGAAGACCCCAGTGACAG (forward) and TAATTAGGATCCAATAGCCGTCTTGGCAGTC (reverse). The MEPE probe was a 1.45 kbp, exon 5, cDNA insert corresponding to the last exon of human MEPE which constitutes 95% of the mature protein as defined by Rowe et al. (21). The last exon was amplified by PCR from human genomic DNA using a 5' oligonucleotide with a NdeI restriction site engineered in (AGTACCCATATGAAAGACAATATTGGTTTTCA-CCAT) and a 3' oligonucleotide with a BamHI site (CTGATGGGATCCCTAGTCAC-CATCGCTCTCAC). The PCR product was subcloned into pBluescript, sequenced, and the ~1.5 kbp insert released with NdeI plus BamHI and labeled. The DSPP probe corresponding to the last exon was similarly amplified using a 5' oligonucleotide with a HindIII restriction site engineered in (CTGTTGGTACCGATATCGAAATCAAGGGT-CCCAGCAG) and a 3' oligonucleotide with a KpnI restriction site (GTGCAAAGCTT-CTAATCATCACTGGTTGAGTGG) subcloned, sequenced and the released ~2.6 kbp insert labeled.

Matrix metalloproteinase probes.

Specific probes of ~300 bp each for human MMP-2, MMP-3 and MMP-9 were made by PCR using human genomic DNA as template and the following oligonucleotides. MMP-2: ATTAGGATCCGGTCACAGCTACTTCTTCAAG (forward with BamHI site added for subcloning) and ATATGGATCCGCCTGGGAGGAGT-ACAG (reverse with BamHI site), MMP-3: ATATGGATCCAGCTGGCTTAATTGT-TGAAAG (forward with BamHI) and TAATGGATCCAACACTGACAAATCGTCTT-TATTA (reverse with BamHI). MMP-9: AATTGAATTCAGAGAAAGCCTATTTCT-

GCCAG (forward with EcoRI) and TAATGAATTCGGTTAGAGAATCCAAGTTA-TTAG (reverse with EcoRI). In each case the PCR products were subcloned into pBluescript, verified by sequencing, and the ~0.3 kbp inserts released and labeled. Membranes were used up to three times, each time removing the previous probe according to the manufacturer's instructions. The stripped membranes were re-imaged by PhosphorImager to verify the removal of the previous probe.

Statistical analysis

Clinical data linked to samples spotted on the cancer profiling array were accessed through the manufacturer's world wide web-based data base (<http://bioinfo.clonetech.com/dparray/array-list-action.do>). Mean five year survival statistics based on stage at time of diagnosis were obtained from the SEER Cancer Statistics Review (27). Comparisons between normal and tumor tissue (derived from the same subject) were performed using a paired t-test. Potential correlations between SIBLING and specific MMP expression were tested by regression analysis. The association of SIBLING expression levels with tumor stage was investigated using both regression and ANOVA analysis. Because of significant differences in standard deviations of group means between different tumor stages, the Kruskal-Wallis nonparametric ANOVA was employed to confirm statistical significance. Spearman correlation of stage and SIBLING expression was performed on untransformed data. All statistical calculations were carried out using StatView software (SAS Institute, Inc.)

RESULTS

SIBLINGs are elevated in multiple cancer types.

Because BSP and OPN protein expression have been found to be greatly increased in many separate, often immunohistochemistry-based studies of different neoplasms, the expression levels of five SIBLING gene family members were determined using a commercial cancer array. The array included normalized cDNA from tumor and corresponding normal tissues from 241 individual patients, as well as certain internal controls (Figure 1). Because the sample sizes were too small for some tumor types on the array, the tissues reported for this study include only breast, uterus, colon, stomach, ovary, lung, kidney, rectum, and thyroid. In each array experiment, the patient's normal and tumor cDNA was separately hybridized with ³²P-labeled probes for BSP, OPN, DMP1, MEPE and DSPP and the array was digitized by PhosphorImager. While BSP, DMP1 and DSPP exhibited minimal normal tissue expression, significant OPN expression by normal tissues was observed. In fact, the highest levels of expression of osteopontin were seen in normal kidney. Because MEPE expression was minimal in both normal and tumor tissue, its expression was not analyzed further (data not shown). The amount of hybridized probe was quantified and the average expression values of BSP, OPN, DMP1, and DSPP in normal and tumor tissue were compared (Figure 2). The expression levels of BSP were significantly elevated (from 2 to 6-fold) in cancer of the breast, colon, rectum, thyroid and kidney. OPN expression was significantly elevated (2 to 4-fold) in cancer of the breast, uterus, colon, ovary, lung, rectum, and thyroid. DMP1 exhibited significant (2 to 3-fold) elevated expression in cancer of the breast, uterus, colon and lung, while DSPP exhibited significant (2-fold) increase in cancer of the breast

and lung. Elevated SIBLING family expression was greatest in breast cancer where four different family members were increased. Colon, lung and thyroid cancer had significantly elevated expression of three different SIBLING family members. Of the nine different types of tumors quantified, each one had a significantly high expression of at least one SIBLING.

MMPs are elevated in multiple cancer types.

We have recently shown that three members of the SIBLING family can specifically bind and modulate the activity of three different MMPs (22). The SIBLINGs BSP, OPN and DMP1 were found to bind to and modulate the activity of MMP-2, MMP-3, and MMP-9, respectively. Corresponding MMP partners for DSPP and MEPE, if any, have yet to be identified. Because MMPs have been postulated to play major roles in tumor cell progression and metastasis (28), the expression levels of SIBLING-matched MMPs were screened in different cancer types. The cancer arrays were separately hybridized with probes for MMP-2, MMP-3 and MMP-9 and the expression values between normal and the corresponding tumor sample for each patient were compared (Figure 3). MMP-2 expression was significantly elevated in cancer of the colon, stomach, lung and rectum. MMP-3 expression exhibited significant elevation in cancer of the breast, colon, stomach and rectum. MMP-9 expression levels were significantly elevated in cancer of the breast, uterus, colon, stomach, ovary, lung, rectum and kidney. The increases in expression ranged from 2- to 3-fold higher for MMP-2 and MMP-3, while expression levels were increased 2- to 7-fold for MMP-9.

Correlated expression of SIBLINGs and their partner MMPs.

Given the observed binding and activation specificity seen with SIBLINGs and their partner MMPs (BSP with MMP-2, OPN with MMP-3, and DMP1 with MMP-9 (22)), it was reasonable to postulate that SIBLINGs and their paired MMPs might exhibit correlated expression levels. When the levels of SIBLING and their matched MMP expressed by individual tumors were analyzed by regression analysis, significant correlation was seen within different cancer types (Figure 4). The expression of BSP and MMP-2 in breast and colon cancer were significantly correlated ($r^2 = 0.40$, $p \leq 0.0001$ and $r^2 = 0.36$, $p \leq 0.0001$, respectively). OPN pairing with MMP-3 showed a significant correlation in stomach and ovary cancer ($r^2 = 0.52$, $p \leq 0.0001$ and $r^2 = 0.45$, $p \leq 0.005$, respectively). DMP1 and MMP-9 expression were significantly correlated in lung and kidney cancer ($r^2 = 0.60$, $p \leq 0.001$ and $r^2 = 0.39$, $p \leq 0.05$, respectively). Mismatched pairs of BSP with MMP-3, OPN with MMP-2, or DMP1 with MMP-2 for example showed no significant correlation (data not shown).

SIBLINGs expression is distinct in different cancer subtypes.

Within cancers arising from a given tissue/organ, there are histo-pathologically defined subtypes that are often used in assessing disease course and treatment. There were sufficient numbers of breast cancer array samples to permit segregation by clinically defined subtypes of ductal versus lobular tumors. The results of microarray screening of SIBLING expression in breast cancer tissue were segregated by the pathological classification and the average values of each group compared (Figure 5A). SIBLING

mRNA levels were significantly higher in the ductal cancer groups, while the levels in the lobular group were intermediate between normal and ductal levels.

A similar analysis was carried out on uterine cancer samples where there were sufficient numbers to permit segregation into clinically defined subtypes of adenocarcinoma, squamous cell and benign tumors (Figure 5B). Osteopontin expression was significantly different between the two subtypes of malignant uterine tumors ($p \leq 0.005$) as well as between malignant and benign tumors ($p \leq 0.05$). The adenocarcinoma subtype expressed higher levels than the squamous cell subtype.

SIBLING expression and tumor stage.

Defined cancer stages represent how large the tumor is and how far it may have spread. The association of SIBLING expression levels with tumor progression was investigated by identifying tumor types with sufficient clinical detail to stratify into different tumor stages. Tumors from colon, rectal and lung cancer were grouped by stage and the distribution of SIBLINGs compared (Figure 6). In general, cancer stages mark tumors that were either localized and had a relatively small size (stage I), localized and larger in size (stage II), metastasized to lymph nodes (stage III), or metastasized to distant sites (stage IV). Colon cancer tumors exhibited mean values of BSP, OPN, DMP1 and DSPP that increased between stage I and stage III. Colon tumors with distant metastases exhibited SIBLING values with a similar or lower pattern of distribution than that of stage III. Rectal cancer tumors showed increasing BSP, OPN and DMP1 levels from stage I to stage IV, while DSPP values were unchanged across different stages. In lung cancer, BSP, OPN and DSPP levels increased with increasing stage. Regression analysis

revealed significant correlation between SIBLING expression and stage (Table I). In rectal tumors, BSP, OPN and DMP1 levels correlated with stage, while for lung cancer, BSP, OPN and DSPP levels correlated with stage. There was no significant correlation between SIBLING expression and tumor stage in colon cancer.

Breast cancer tumors were stratified into TNM stages, which reflect tumor size (T), lymph node involvement (N), and metastatic state (M). Enough breast tumor samples were analyzed to enable multiple analyses of SIBLING expression and tumor progression. Tumors of a similar, small size were compared for SIBLING expression patterns as lymph node status changed (constant T1 with N ranging from 0 to 2). For BSP, OPN, DMP1 and DSPP significant differences were observed for the expression pattern as a function of nodal involvement when analyzed by nonparametric ANOVA (Figure 7 and Table I). Regression analysis of SIBLING values and N stage yielded significant correlation for all four SIBLINGs. The contribution of tumor size to SIBLING expression was next investigated by segregating breast tumors into different T states while nodal involvement and metastatic state were held constant. Significant differences between SIBLING and tumor size were demonstrated by nonparametric ANOVA and regression analysis demonstrated significant correlation between BSP, OPN, DMP1, and DSPP expression and T stage.

SIBLING expression and survival.

Different tumor stages are usually associated with different survival rates. We next investigated whether SIBLING expression correlated with survival. Mean BSP and OPN expression levels for defined cancer stages were plotted versus mean five year survival statistics obtained from the SEER Cancer Statistics Review. For rectal, colon, lung and breast tumors, both mean BSP and mean OPN expression increased with decreasing survival (Figure 8). Regression analysis of mean BSP expression by rectal, colon and lung tumors combined yielded a significant correlation with $r^2 = 0.60$ and $p < 0.005$. For OPN, grouping rectal and colon tumor mean values yielded significant regression correlation with $r^2 = 0.61$ and $p < 0.05$, grouping lung and breast tumor mean values yielded significant regression correlation with $r^2 = 0.78$ and $p < 0.001$. The levels of mean DSPP expression in breast and lung cancer were also correlated with survival (data not shown).

DISCUSSION

Retrospective studies of pathological specimens have shown that BSP expression was associated with poor survival in breast cancer (13) and prostate cancer (17) while OPN expression was associated with clinical severity in lung cancer (29), lymph node negative breast cancer (16), gastric cancer (30), and perhaps ovarian carcinoma (31). The current microarray results demonstrate elevated expression of BSP, OPN, DMP1 and DSPP mRNA in multiple types of cancer. A correlation of SIBLING message expression levels with MMP message levels of their partners (BSP with MMP-2, OPN with MMP-3, and DMP1 with MMP-9) was observed. That, in association with the recently described ability of these SIBLINGs to bind to and modulate the activity of specific MMPs, suggests that the same factors that activate SIBLING genes in tumor progression may be the same ones that can activate the corresponding MMP genes. It is also possible that the expression of one SIBLING member in a tumor may induce the production of its corresponding MMP partner, or vice versa. Interestingly, SIBLING production by tumors could facilitate angiogenesis, as both BSP and OPN have been shown to possess angiogenesis activity *in vivo* (32, 33).

The observed increase in MMP-2 expression observed in tumor samples is consistent with previous studies of breast (34, 35), colon (36-40), stomach (41, 42), lung (43-46), rectal (36, 47), and kidney cancer (48-50). While a strong association of increased MMP-3 has been found in breast cancer (34, 51-54), the increased expression levels observed in other tumor types is not as well supported by published literature.

Altered MMP-3 levels have been observed in colon (55-57), stomach (58-60), and rectal (61) cancer, though in some cases the increases were relatively small. In addition, studies have indicated that the MMP-3 source was not necessarily tumor cell but stromal or another infiltrating cell type, distinct from the tumor. The observed increases in MMP-9 expression are consistent with published studies of breast (34, 62), uterine (63, 64), colon (39, 46, 65), stomach (66-68), ovary (69, 70), lung (43, 71), rectal (36, 72), and kidney cancer (49, 73).

SIBLING expression was different between different subtypes of cancer. While the historical basis for the distinction between the main two types of breast cancer (the belief that ductal carcinomas arose from ducts and lobular carcinomas from lobules) is subject to debate (both can arise from the terminal duct lobular unit), there is evidence that the two classes as used clinically refer to disease entities that differ in tumor size, shape, dissemination and proliferation rates (74). The most common hallmark associated with the lobular classification is multifocality. Lobular tumors tend to be more slowly proliferating than ductal tumors. They also tend to frequently exhibit hormone receptor-positivity and show distinct chromosomal changes (75, 76). The more rapidly progressing ductal tumors had an associated higher level of SIBLING expression. OPN was recently identified by microarray analysis as a discriminating marker between ductal and lobular cancer (77). In our current study, OPN, as well as BSP, DMP1 and DSPP were significantly different between lobular and ductal tumors. Similarly, the association of higher OPN expression with adenocarcinomas as opposed to squamous cell carcinomas in uterine cancer may be associated with different size, shape and progression rates.

SIBLING expression correlated with tumor stages associated with changing size and lymph node involvement. These observations are consistent with SIBLING expression coupled with MMP activity modulation having an effect on early tumor progression. These results suggest SIBLINGs as potential markers of early disease progression in a number of different cancer types which currently lack clinical markers (e.g. ovarian tumors). Future studies of SIBLING expression and serum levels will address the degree to which these tumor biomarkers can be correlated with disease progression.

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FOOTNOTES

The abbreviations used are: SIBLINGS, small integrin binding ligand N-linked glycoproteins; MMP, matrix metalloproteinase; BSP, bone sialoprotein; OPN, osteopontin; DMP1, dentin matrix protein-1; MEPE, matrix extracellular phosphoglycoprotein; DSPP, dentin sialophosphoprotein; TIMPs, tissue inhibitors of matrix metalloproteinases;

TABLES

Table I. SIBLING expression and tumor staging.

	BSP	OPN	DMP1	DSPP
colon cancer ANOVA ^a	p < 0.0001	p < 0.005	n.s.	p < 0.05
rectal cancer ANOVA	n.s.	p < 0.0001	n.s.	n.s.
lung cancer ANOVA	p < 0.001	p < 0.005	n.s.	p < 0.05
colon tumor stage correlation ^b	n.s.	n.s.	n.s.	n.s.
rectal tumor stage correlation	r ² = 0.19 p < 0.05	r ² = 0.42 p < 0.0001	r ² = 0.20 p < 0.05	n.s.
lung tumor stage correlation	r ² = 0.65 p < 0.0001	r ² = 0.68 p < 0.05	n.s.	r ² = 0.61 p < 0.0001
breast tumor T stage correlation ^c	r ² = 0.33 p < 0.001	r ² = 0.18 p < 0.05	r ² = 0.18 p < 0.05	r ² = 0.40 p < 0.0005
breast tumor N stage correlation ^d	r ² = 0.83 p < 0.0001	r ² = 0.28 p < 0.05	r ² = 0.48 p < 0.005	r ² = 0.31 p < 0.05

^a Analysis of variance comparison of SIBLING expression across four distinct tumor stages.

^b Correlation between mean SIBLING values and tumor stage. Tumor stages for colon, rectal and lung cancer were defined as stated in the figure legend to Figure 5.

^c Correlation between mean SIBLING values and T stage. Lymph node and distant metastasis stages were constant (N0 and M0). Breast tumor T stages were defined as stated in the figure legend to Figure 6.

^d Correlation between mean SIBLING values and N stage. Tumor size and distant metastasis stages were constant (T1 and M0). Breast tumor N stages were defined as stated in the figure legend to Figure 6.

FIGURE LEGENDS

Figure 1. SIBLING expression in different cancer types. A cancer profiling array was hybridized with cDNA probes for SIBLINGs. The arrays contained samples from 13 different types of cancer with paired normal and tumor tissue mRNA from individual subjects (A). The amount of hybridized probe for BSP (B), OPN (C), and DMP1 (D) was visualized by PhosphorImager. Abbreviations: br, breast cancer; ut, uterine cancer; co, colon cancer; st, stomach cancer; ov, ovarian cancer; lu, lung cancer; ki, kidney cancer; re, rectal cancer; th, thyroid cancer; n, normal tissue; t, tumor tissue. Those hybridization spots that are not contiguous with the identified tumor types represent patient samples with tumor types too few in number to be statistically useful.

Figure 2. SIBLING mRNAs are induced in multiple cancer types. Digitized exposures from Figure 1 were quantified using ImageQuant software and the mean values of relative expression of BSP (A), OPN (B), DMP1 (C), and DSPP (D) in normal tissue (white bars) and tumor tissue (black bars) determined for each of nine different cancer types. Asterisks denote the statistical significance as determined by paired t-tests where *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0001$. Error bars represent the standard error of the mean and numbers in parenthesis represent number of subjects. OPN expression in both normal and tumor tissue from kidney is shown at $1/10^{\text{th}}$ the actual mean values.

Figure 3. MMP mRNAs are induced in multiple cancer types. Cancer profiling arrays were hybridized with cDNA probes for different MMPs, the amount of hybridized probe was quantified using ImageQuant software and the mean values of expression of MMP-2 (A), MMP-3 (B) and MMP-9 (C) in normal tissue (white bars) and tumor tissue (black bars) determined for each of nine different cancer types. Asterisks denote the statistical significance as determined by paired t-tests where *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0001$. Error bars represent the standard error of the mean and numbers in parenthesis represent number of subjects.

Figure 4. Paired SIBLING and MMP expression is correlated in specific cancers. The expression levels of SIBLINGs and their respective binding partner MMPs were analyzed by regression analysis. BSP and MMP-2 levels in breast (A) and colon cancer (D), OPN and MMP-3 levels in stomach (B) and ovary cancer (E), as well as DMP1 and MMP-9 levels in lung (C) and rectal cancer (F) were paired by subject and analyzed by regression analysis.

Figure 5. SIBLING expression distinguishes cancer subtypes for breast and uterine tumors. The expression values of BSP, OPN, DMP1, and DSPP by breast cancer tumors were stratified by pathological classification (ductal versus lobular) and the average values compared (A). Similarly, the expression of SIBLINGs by uterine tumors stratified into groups defined as adenocarcinoma, squamous cell or benign tumor, were averaged and compared (B). Asterisks denote the statistical significance as determined by t-test

where *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$. Error bars represent the standard error of the mean.

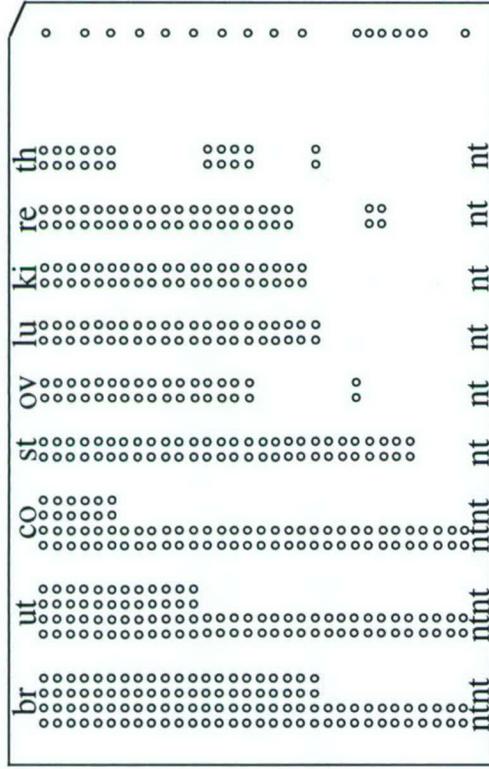
Figure 6. Comparison of SIBLING mRNA levels with tumor stage in colon, rectal and lung cancer. The expression values of BSP (A, B, C), OPN (D, E, F), DMP1 (G, H, I), and DSPP (J, K, L) by colon cancer tumors (A, D, G, J), rectal cancer tumors (B, E, H, K), and lung cancer tumors (C, F, I, L) were stratified by pathological classification (stage) and the average values compared. Top, bottom and line through the middle correspond to 75th percentile, 25th percentile and 50th percentile (median), respectively. Error bar whiskers represent the 10th and 90th percentile, while the solid square symbol indicates the arithmetic mean. For rectal and colon cancer stages: I, tumor invaded submucosa; II, tumor invaded through muscularis propia; III, invasive tumor with metastasis in 1 to 3 pericolic or perirectal lymph nodes; IV, invasive tumor with metastasis in pericolic or perirectal lymph nodes and distant metastasis. For lung cancer stages: I, tumor < 3 cm in greatest dimension; IB, tumor > 3 cm in greatest dimension, involved main bronchus, associated with atelectasis or obstructive pneumonitis; II, metastasis to ipsilateral peribronchial and/or ipsolateral lymph nodes; III, metastasis to ipsilateral mediastinal, and/or subcarinal lymph nodes. The number of subjects (n) for each group is shown at the bottom.

Figure 7. Comparison of SIBLING mRNA levels and tumor stage in breast cancer. The expression values of BSP (A, B), OPN (C, D), DMP1 (E, F), and DSPP (G, H) by breast cancer tumors were stratified by TNM stage and the average values compared. Top,

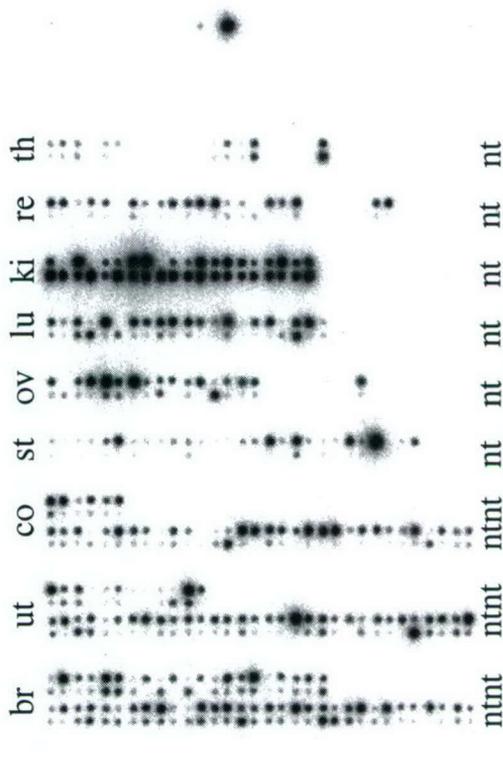
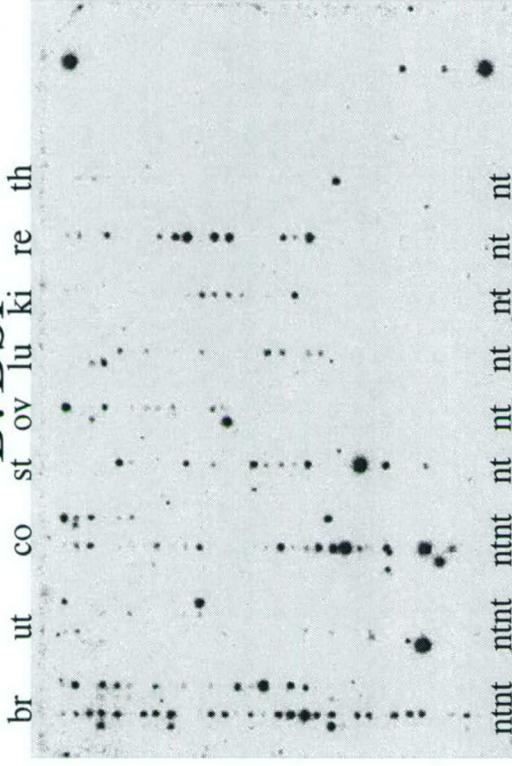
bottom and line through the middle correspond to 75th percentile, 25th percentile and 50th percentile (median), respectively. Error bar whiskers represent the 10th and 90th percentile, while the solid square symbol indicates the arithmetic mean. For breast cancer TNM staging: T1, tumor \leq 2 cm in greatest dimension; T2, 2 cm < tumor < 5 cm; T3, tumor > 5 cm; N0, no regional lymph node metastasis; N1, metastasis to movable ipsilateral axillary lymph node(s); N2, metastasis to movable ipsilateral axillary lymph node(s) fixed to one another or to other structure; M0, no distant metastasis; M1, distant metastasis. The number of subjects (n) for each group is shown at the bottom.

Figure 8. SIBLING mRNA expression levels correlate with survival. Mean BSP and OPN expression levels for 4 distinct cancer stages (I, II, III, and IV for colon and rectal tumors; I, IB, II and II for lung tumors) were plotted versus mean five-year survival for those specific cancer types and stages. Mean five year survival statistics based on stage at time of diagnosis were obtained from the SEER Cancer Statistics Review.

A. array key



B. BSP

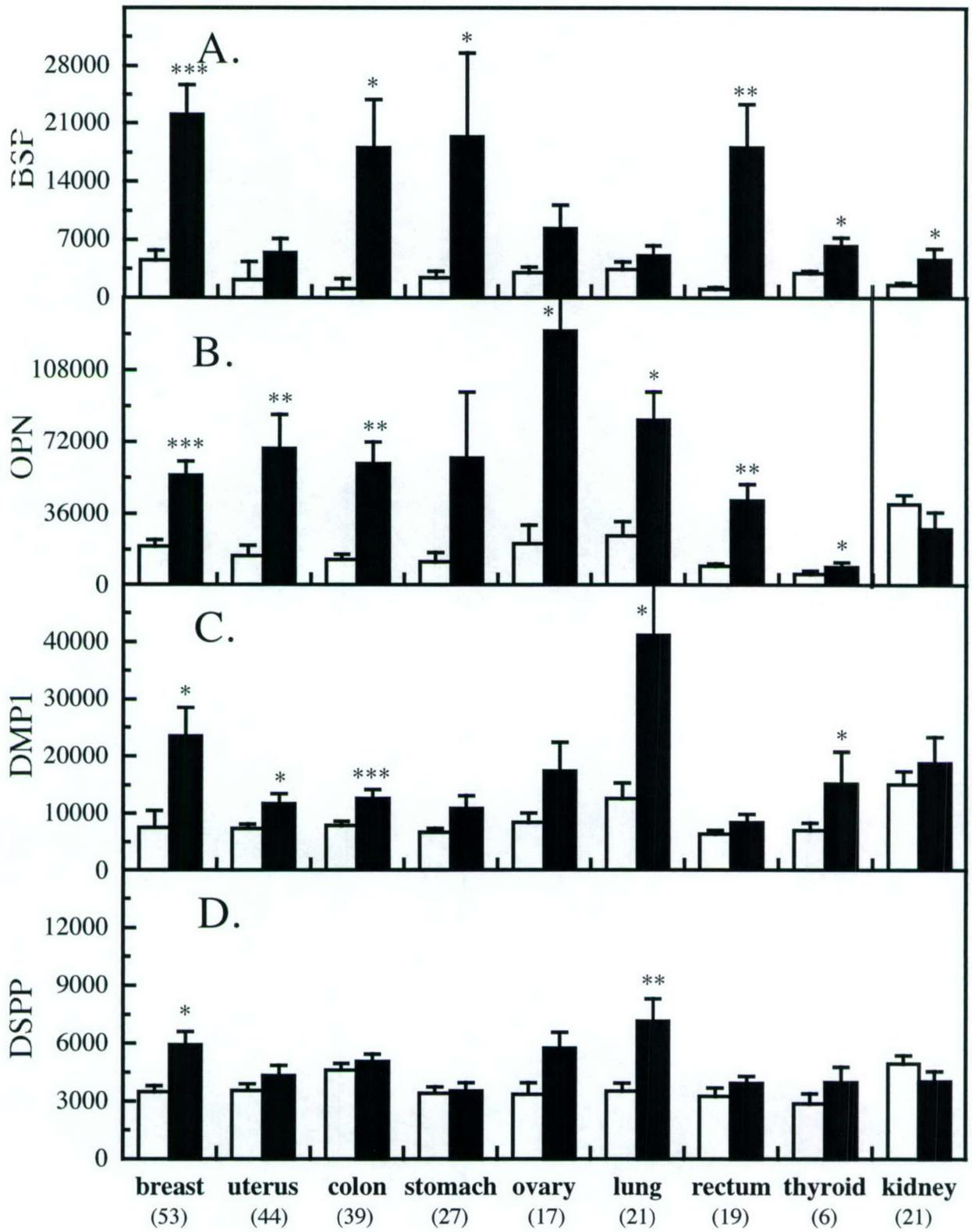


C. OPN

D. DMPI

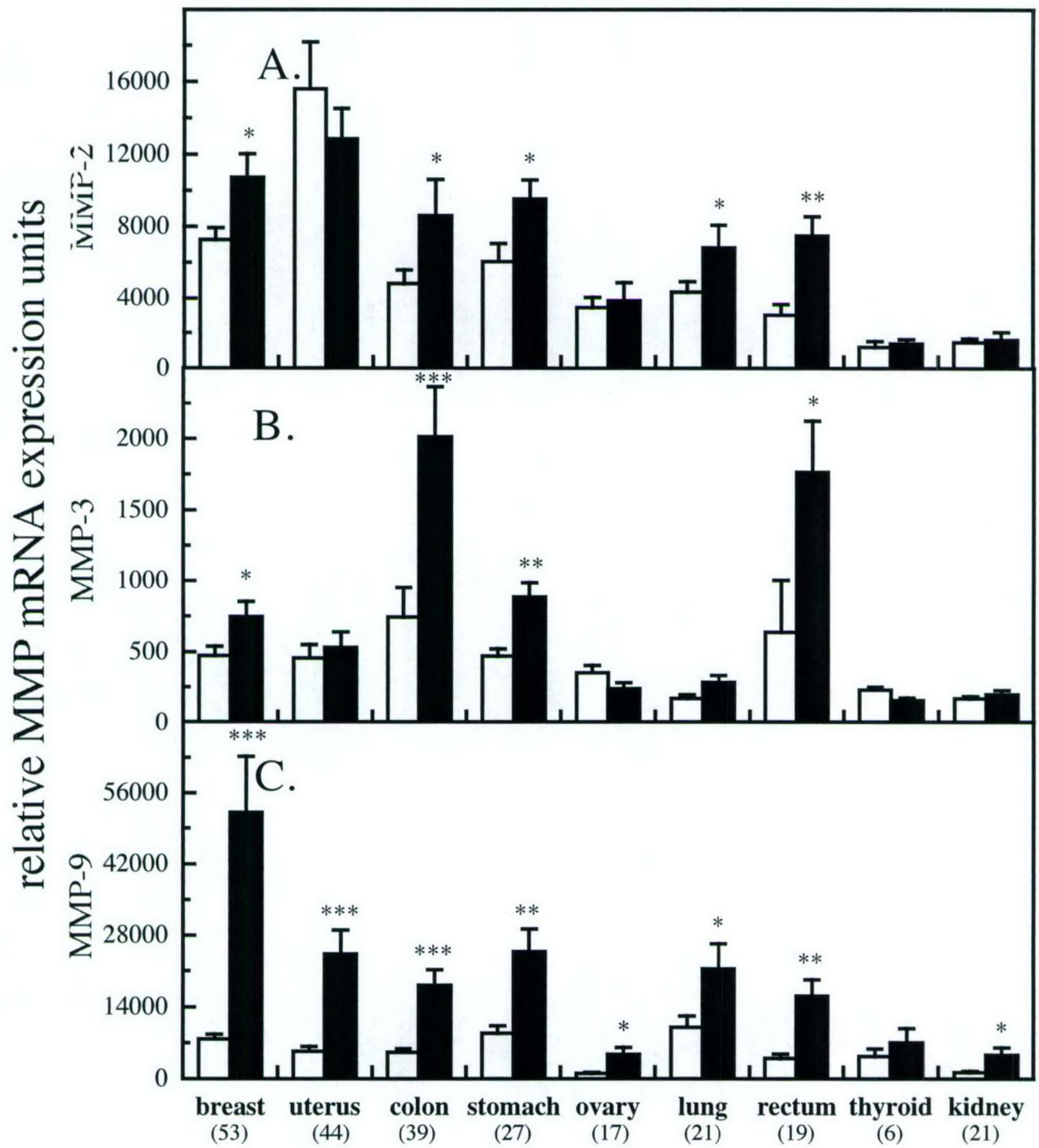
Figure 1

relative SIBLING mRNA expression units



* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0001$

Figure 2



* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0001$

Figure 3

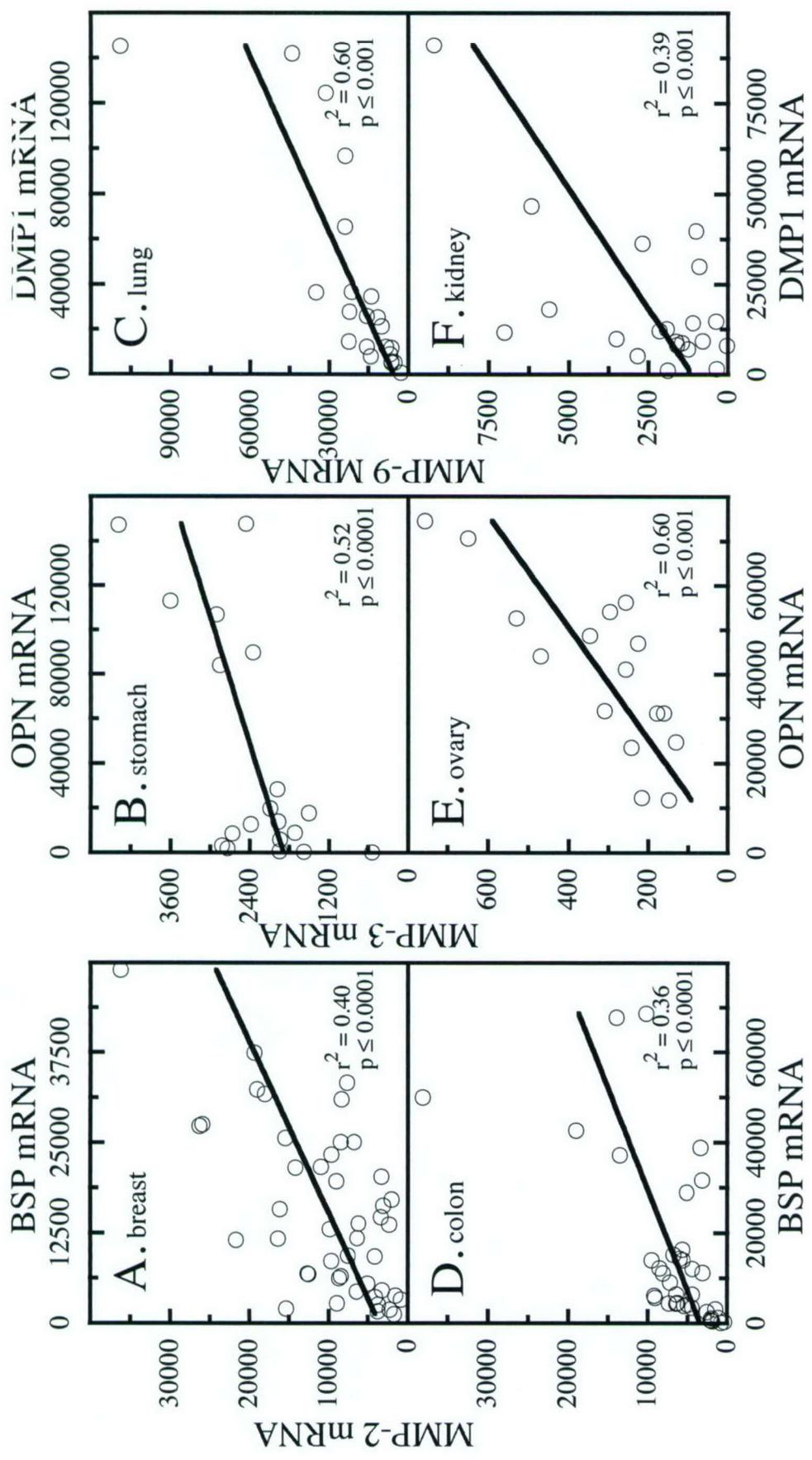


Figure 4

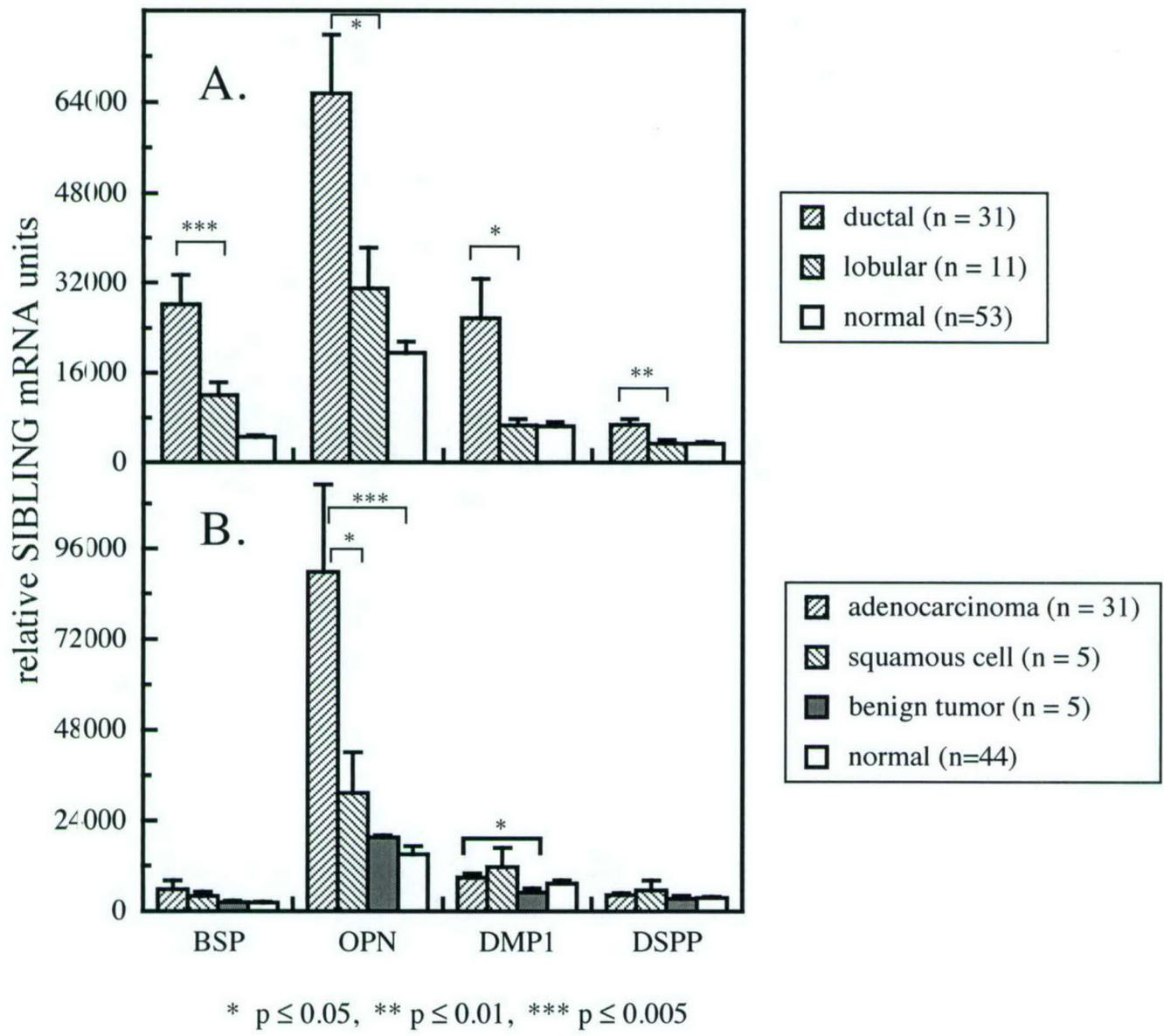


Figure 5

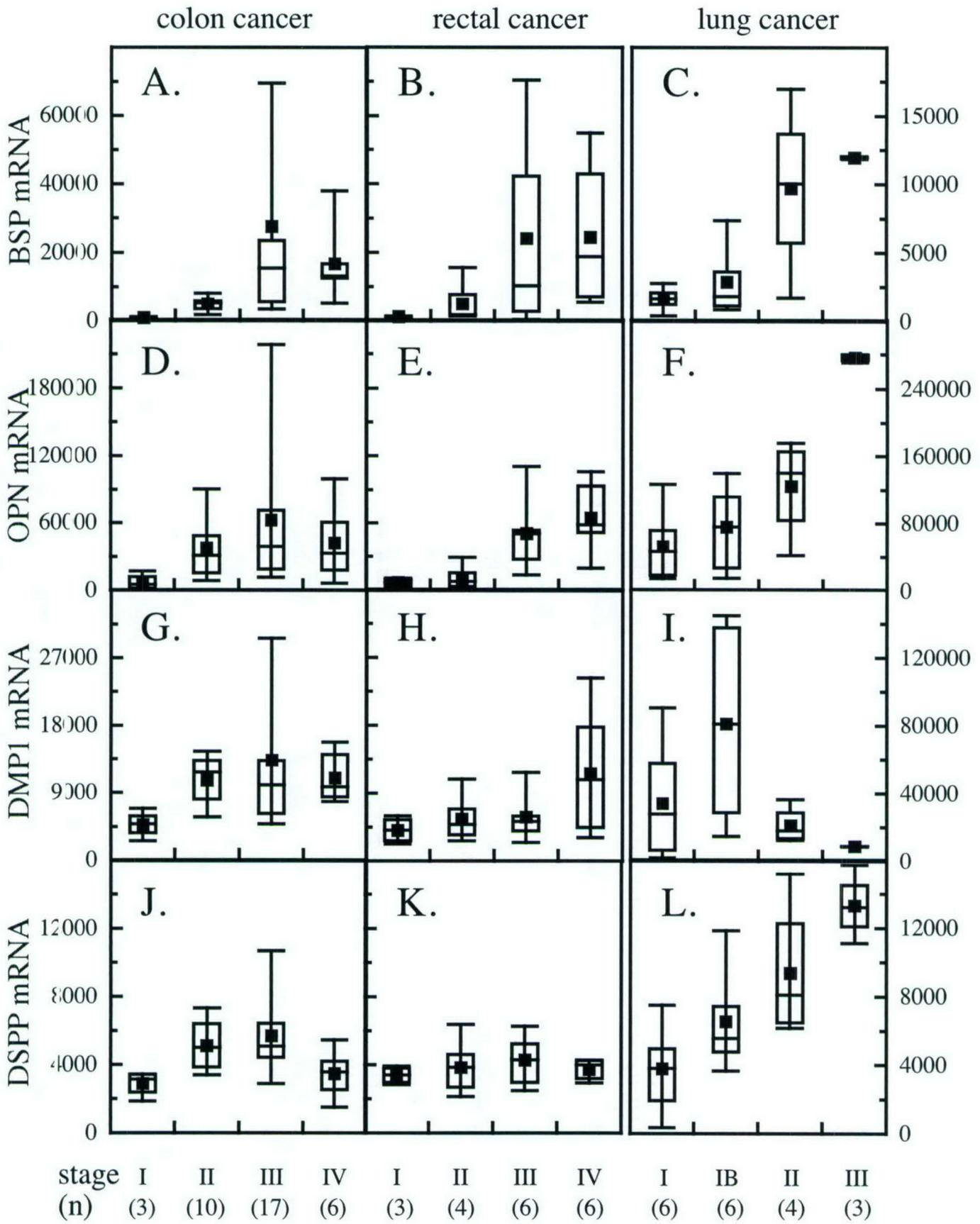
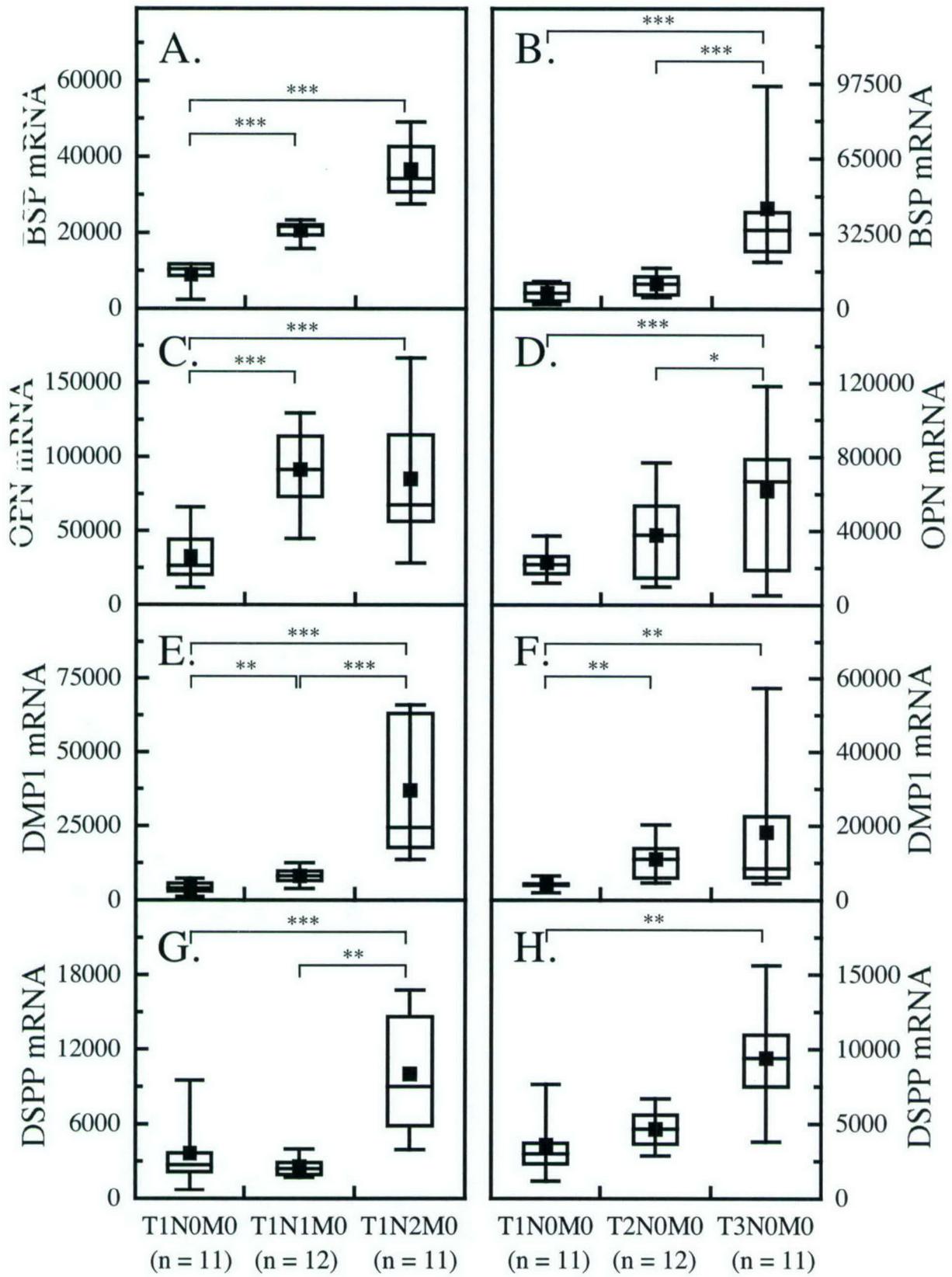


Figure 6



* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001,

Figure 7

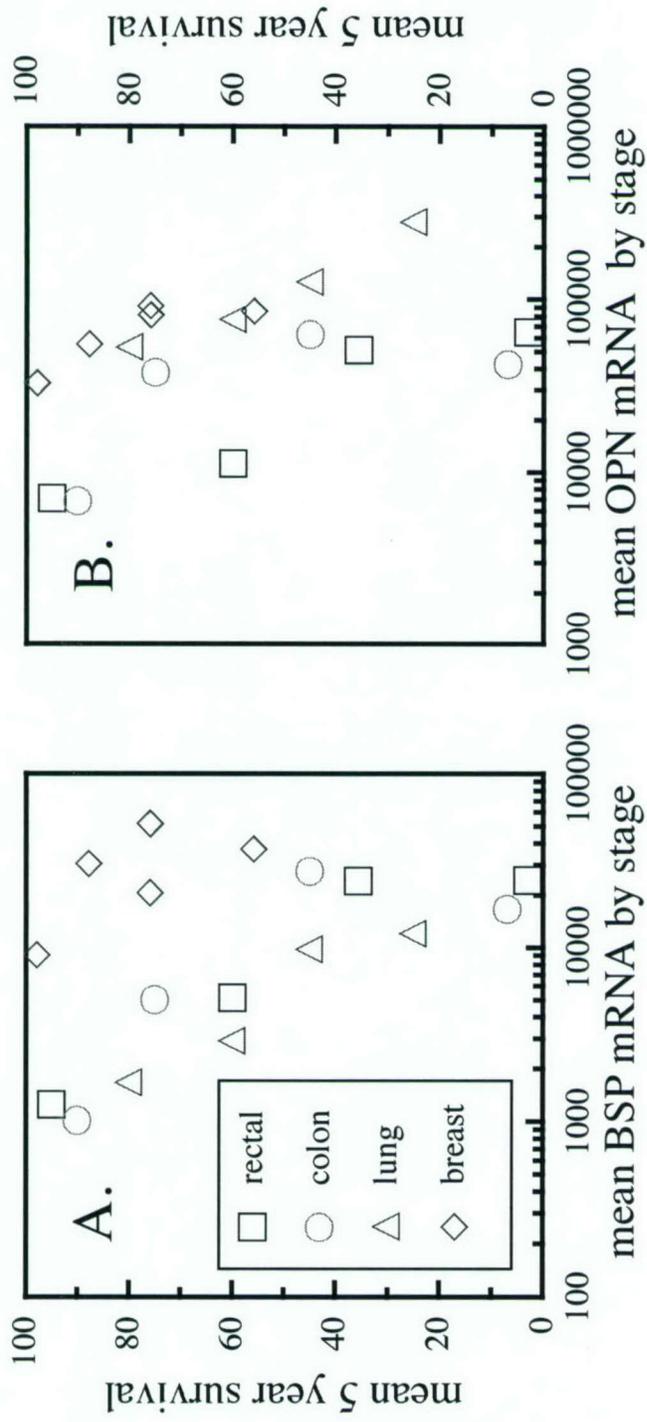


Figure 8